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ACTIVATION OF SKELETAL MUSCLE GLUCOSE UPTAKE BY AN AMINO ACID MIXTURE AND ITS IMPACT ON GLUCOSE TOLERANCE AND INSULIN RESISTANCE

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by

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Dedication

To my family.

In memory of Henry J. Herr.

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Recent research suggests that amino acids can significantly increase skeletal muscle glucose uptake. However, the mechanism(s) have not been fully elucidated and it is also not clear if the beneficial impact amino acids have on healthy tissue translates to insulin resistant skeletal muscle. Therefore, in this series of studies, the effects of an amino acid mixture on glucose tolerance and insulin resistance were investigated. Study 1 Experiment-1 (Exp-1) demonstrated that an amino acid mixture significantly reduced the blood glucose response to an oral glucose challenge in Sprague Dawley rats. In Study 1 Exp-2, it was found that the improved glucose tolerance was due to an increase in skeletal muscle glucose uptake. The enhanced amino acid induced muscle glucose uptake was associated with improved cellular signaling. In Study 1 we could not determine the combined and/or individual effects of insulin and amino acids on glucose uptake, so in Study 2, the hindlimb of Sprague Dawley rats were perfused with glucose with or without amino acids in the presence and absence of insulin. Study 2, confirmed

our previous findings that an amino acid mixture increased skeletal muscle glucose uptake compared to a carbohydrate supplement in the presence of insulin. The enhanced amino acid-stimulated glucose uptake was not due to increased phosphatidylinositol 3-kinase (PI 3-kinase) activity, although it was related to an increase in Akt substrate of 160 kDa (AS160) phosphorylation and a greater number of glucose transporters at the plasma membrane. In the final experiment, Study 3 investigated whether amino acids could improve glucose tolerance in an insulin resistant model. Study 3 Exp-1, demonstrated that an amino acid mixture significantly lowered the blood glucose response to an oral glucose challenge in obese Zucker rats. Study 3 Exp-2 showed that the improved glucose tolerance was due to enhanced amino acid induced skeletal muscle glucose uptake. Taken together, the results of this research suggests that adding an amino acid mixture to a carbohydrate supplement improves the blood glucose response to an oral glucose challenge, acutely lowers insulin resistance and this appears due to increased skeletal muscle glucose clearance and enhanced cellular signaling.

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Chapter I: General Introduction

Under normal conditions, the acute ingestion of carbohydrate elevates blood glucose levels, which stimulates pancreatic insulin secretion. The hormone insulin returns blood glucose back to fasting levels by stimulating peripheral tissue uptake and limiting its release from the liver. Thus, for proper blood glucose homeostasis, there must be a balance between the entry of glucose into the blood, its removal by peripheral tissues and its output from the liver. Of the insulin sensitive tissues, skeletal muscle serves as the primary site of whole body glucose disposal. An estimated 80% of all insulin-stimulated glucose uptake can be attributed to skeletal muscle, with the majority of this glucose at rest being converted and stored as glycogen for later energy use (2). Despite the fact that glucose is the primary fuel for many of the body's functions, low levels can be fatal and high blood glucose levels, when left unmanaged for an extended time, can lead to morbid obesity, blindness, cardiovascular disease and peripheral neuropathy. Therefore, blood glucose homeostasis is a highly regulated process. Although this is a very effective process in many people there is a rapidly growing segment of the population in which this process begins to fail, resulting in dangerously high glucose and insulin levels.

Insulin resistance is a condition in which the cells of the body do not respond appropriately, or are resistant to the effects of insulin. While the rise in blood glucose is quickly returned to baseline by insulin in healthy individuals, the same amount of insulin has little effect in lowering blood glucose in insulin resistant people. Thus, normal amounts of insulin secreted from the pancreas are unable to produce a normal blood glucose response. As a compensation mechanism, the pancreas is forced to release more

insulin in order to return blood glucose to baseline levels. The inability to properly remove blood glucose can be attributed to reduced skeletal muscle glucose transport (1). Glucose transport is defined as the penetration of glucose across the plasma membrane. This is a reversible process and the net flux of glucose depends on both the concentration gradient and the number of glucose transporters at the plasma membrane (5). In contrast, the irreversible removal of glucose from the extracellular space and into the cell is defined as glucose uptake.

Recent studies suggest that amino acids may aid in the regulation of blood glucose. Of these, both isoleucine and leucine are the amino acids in which much of the research has focused on. Although these amino acids have been shown to reduce blood glucose, many questions regarding their mechanism(s) of action have not been answered. Studies have reported that amino acids increase both *in vitro* (3, 7, 8) and *in vivo* (3, 4, 6) skeletal muscle glucose uptake. Nishitani et al. (8) demonstrated using an isolated soleus muscle preparation that both phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase C (PKC), but not mammalian target of rapamycin (mTOR), was directly involved with leucine-stimulated glucose uptake. In agreement with these findings, Doi et al. (3) reported that isoleucine-stimulated glucose uptake in C_2C_{12} myotubes was inhibited in the presence of a specific inhibitor for PI 3-kinase but not in the presence of an inhibitor of mTOR. Although there is strong evidence suggesting amino acids can increase glucose uptake in healthy skeletal muscle, it is not clear whether these same amino acids have an affect in insulin resistant tissue.

Therefore, this series of studies aimed to investigate the effects of a novel amino acid mixture on glucose tolerance and insulin resistance. In doing so, these studies will assess differences in blood glucose, plasma insulin, skeletal muscle glucose uptake and

signaling proteins between a supplement containing only carbohydrate and a carbohydrate plus amino acid mixture.

OBJECTIVES

Study 1: Study 1 consists of 2 separate experiments. The purpose of experiment-1 (Exp-1) is to evaluate the ability of an amino acid supplement containing a mixture of branched-chain and essential amino acids to improve glucose tolerance in Sprague Dawley rats during an oral glucose tolerance test (OGTT). This experiment is designed to determine 1) if orally consuming a carbohydrate supplement with an amino acid mixture improves glucose tolerance, 2) if adding amino acids to a carbohydrate load alters circulating insulin levels and 3) how increasing the leucine concentration of the amino acid mixture will affect glucose tolerance. The purpose of experiment-2 (Exp-2) is to determine if differences in glucose tolerance observed in Exp-1 are associated with changes in skeletal muscle glucose clearance. This experiment is designed to determine 1) if the amino acid mixture improves the glucose response during an OGTT via an increase in skeletal muscle glucose uptake and 2) the effect of the amino acid mixture on components of the PI 3-kinase and mTOR signaling pathways.

Study 2: The purpose of study 2 is to investigate the effects of an amino acid supplement containing a mixture of branched-chain and essential amino acids on glucose uptake via the hindlimb perfusion technique in Sprague Dawley rats. This study is designed to determine 1) if perfusing the amino acid mixture increases skeletal muscle insulin-stimulated and non-insulin-stimulate glucose uptake, 2) if amino acids activate PI 3-kinase and 3) if the amino acid mixture enhances cellular signaling.

Study 3: Study 3 consists of 2 separate experiments. The purpose of experiment-1 (Exp-1) is to investigate the effects of an amino acid supplement containing a mixture

of branched-chain and essential amino acids on glucose tolerance and insulin resistance in obese Zucker rats. This experiment is designed to determine 1) if orally consuming a carbohydrate supplement with an amino acid mixture improves glucose tolerance in an insulin resistant model, 2) if adding amino acids to a carbohydrate load alters circulating insulin levels and 3) how increasing the leucine concentration of the amino acid mixture will affect glucose tolerance and insulin resistance. The purpose of experiment-2 (Exp-2) is to determine if differences in glucose tolerance observed in Exp-1 are associated with changes in skeletal muscle glucose clearance. This experiment is designed to determine 1) if the amino acid mixture improves the glucose response during an OGTT via an increase in skeletal muscle glucose uptake and 2) the effect of the amino acid mixture on components of the PI 3-kinase and mTOR signaling pathways.

HYPOTHESIS

Study 1:

1. In comparison with a carbohydrate supplement, addition of the amino acid mixture will increase insulin secretion and decrease the blood glucose response during an oral glucose challenge.
2. Increasing the leucine concentration of the amino acid mixture will further enhance the affects of the supplement.
3. In comparison with a carbohydrate supplement, addition of the amino acid mixture will increase skeletal muscle glucose uptake and this will be associated with increased activation of signaling proteins.

Study 2:

1. In comparison with a perfusate including carbohydrate, adding the amino acid mixture will increase insulin and non-insulin-stimulated glucose uptake.

2. In comparison with a perfuste with carbohydrate, adding the amino acid mixture will increase PI 3-kinase activity.
3. The increase in glucose uptake will be associated with an increase in Akt substrate of 160 kDa (AS160) phosphorylation and plasma membrane glucose transporter 4 (GLUT4) protein concentration.

Study 3:

1. In comparison with a carbohydrate supplement, addition of the amino acid mixture will increase insulin secretion and decrease the blood glucose response during an oral glucose challenge in an insulin resistant model**.**
2. Increasing the leucine concentration of the amino acid mixture will further enhance the affects of the supplement.
3. In comparison with a carbohydrate supplement, addition of the amino acid mixture will increase skeletal muscle glucose uptake in an insulin resistant model and this will be associated with increased activation of signaling proteins.

SIGNIFICANCE

The prevalence of obesity, insulin resistance and type 2 diabetes mellitus (T2D) has reached epidemic proportions. For example, the percentage of Americans who are classified as obese has doubled since 1980, and similar trends have been reported for insulin resistance and T2D. Particularly startling is that T2D, generally viewed as a disease that primarily affects adults, is now being diagnosed in children and teens at an alarming rate. We cannot ignore the fact that genetics are an underlying factor in insulin resistance and T2D in some individuals, but for many people, poor dietary habits and lack of physical activity are the two major components in the development of this disease. Moderating one's diet and prescribing an exercise regime have both been shown to

prevent and/or reverse the effects of insulin resistance by heightening the sensitivity of skeletal muscle to insulin. However, the mechanism(s) for this improvement is unclear. Thus, novel means to improve the regulation of blood glucose is an important area of research because future efforts will increase our understanding of the disease and help identify potential protein targets for intervention.

A single bout of exercise has been shown to improve insulin sensitivity in insulin resistant and T2D subjects. Exercise training offers even greater benefits, with the positive affects being sustained for a much longer period of time. However, those with impaired glucose tolerance may be unable, or unwilling, to engage in regular physical activity. Therefore, dietary factors become even more critical. With the recent movement towards non-pharmacological approaches to medicine, many people have turned toward natural alternatives, such as dietary supplements to improve one's general well being. It is often touted as a more natural, and safer alternative to pharmaceuticals, were there is tradeoff between benefits and side effects. Diabetics are often prescribed a list of medications to help them properly regulate their blood glucose levels. Therefore, those with insulin resistance and diabetes could benefit greatly from a dietary supplement regime that could possibly replace their medications.

It has been known for many years that amino acids are integral in many physiological processes, most notably protein synthesis. However, it has only been recently that amino acids have been demonstrated to play a role in blood glucose homeostasis. Much of the literature regarding the effects of amino acids on blood glucose levels have focused on single amino acids. As a result of these studies, it has been shown that not all amino acids have the same affect on blood glucose levels. For example, isoleucine has been shown to increase glucose uptake. Leucine has also been shown to increase glucose uptake, but this amino acid also increases insulin secretion and

glycogen synthesis. Nevertheless, this raises an intriguing possibility that a mixture containing amino acids with different affects on glucose metabolism could be designed with the goal of improving blood glucose levels.

Therefore, the goal of these studies were to demonstrate that a mixture of amino acids consisting of leucine, isoleucne, valine, methionine and cysteine could lower the blood glucose response to an oral glucose challenge, reduce insulin resistance and provide a mechanism for these improvements. Ideally, the results of these studies could lead to a non-pharmacological based approach to maintaining blood glucose within the proper range.

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Chapter II: Review of the Literature

It is well known that amino acids are essential for protein synthesis. However, recent research suggests that amino acids may also reduce the blood glucose response to an oral glucose challenge. Several investigators have reported that amino acids improve glucose tolerance using both *in vitro* (17, 35, 36) and *in vivo* (17, 18, 28) models. Therefore, this review will discuss the relevant literature regarding insulin signaling, protein/amino acid supplementation and insulin resistance in skeletal muscle.

THE PHOSPHATIDYLINOSITOL 3-KINASE SIGNALING PATHWAY

A rise in blood glucose levels stimulates the release of insulin from the pancreatic β-cells. Because insulin is a peptide hormone it requires a specialized receptor on the cell's surface to exert is actions. The insulin receptor consists of two alpha- and two beta-subunits. The alpha-subunits are located on the extracellular portion of the plasma membrane while the beta subunits span the membrane with both extracellular and intracellular portions. The binding of insulin to the extracellular alpha-subunit causes a conformational change that initiates the autophosphorylation of the beta-subunits. Next, the activated beta-subunits phosphorylates the downstream insulin receptor substrate-1 (IRS-1). In skeletal muscle, both insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) are expressed (51). However, IRS-1 is the primary isoform responsible for insulin-stimulated glucose transport. Once phosphorylated IRS-1 acts a docking protein for molecules containing Src homology 2 (SH2), such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). The docking and binding of IRS-1 to the p85 subunit activates the p110 catalytic subunit of PI 3-kinase. The PI 3-kinase subunit, p110, can then disassociates from p85 and translocate to the plasma membrane, where it

catalyses the conversion of phosphatidylinositol (3,4)-biphosate (PIP2) to phosphatidylinositol (3,4,5)-triphosate (PIP3). The activation of PI 3-kinase is a necessary step in insulin-stimulated glucose transport as has been demonstrated by blocking its actions with an inhibitor. Wortmannin, a specific inhibitor of PI 3-kinase, blocks its kinase activity by binding to the lysine 833 residue of PI 3-kinase causing a conformational change of the protein. Following the administration of wortmannin, Wojtaszewski et al. (52) observed a dose dependent reduction in insulin-stimulated glucose uptake and transport, but the binding of insulin to its receptor, IR-β subunit autophosphorylation and tyrosine phosphorylation of IRS-1 were unaltered. Furthermore, Kanai et al. (29) observed dose dependent inhibition of PI 3-kinase activity and glucose transporter 4 (GLUT4) translocation in response to wortmannin. The author also reported no change in the proximal signaling proteins. Collectively, these findings suggest that PI 3-kinase is required for insulin-stimulated GLUT4 translocation and glucose transport and that the effects of wortmannin were elicited on PI 3-kinase alone.

As discussed above, the activation of PI 3-kinase generates the phosphoinositide lipid PIP3 at the plasma membrane. These membraneous lipids are required for the activation of proteins containing pleckstrin homology domains, including phosphoinositide dependent kinases 1 and 2 (PDK-1 and PDK-2). PDK-1 and PDK-2 activate the downstream serine/threonine kinases protein kinase B (Akt/PKB) and atypical protein kinase C (aPKC). PDK-1 phosphorylates Akt on threonine 308 while serine 473 is phosphorylated by PDK-2, which has recently been identified as mTOR-RICTOR complex (39). Skeletal muscle expresses three forms of Akt/PKB isoforms; 1, 2 and 3. However, it appears that Akt/PKB 2 is the primary isoform involved in skeletal muscle insulin-stimulated glucose uptake. In addition, there are two aPKC isoforms expressed in skeletal muscle, of which PDK-2 phosphorylates aPKC-zeta (aPKC-ζ) on

threonine 410 and aPKC- lambda (aPKC-λ) on threonine residue 403. Both Akt/PKB and aPKC-ζ/λ have been observed to be involved for insulin-stimulated glucose transport in skeletal muscle.

Recently, AS160 has been identified as a potential substrate for Akt/PKB signaling. AS160 contains several Akt/PKB phoshporylaiton sites in addition to a Rab GTPase activating protein (GAP) domain (50). It is believed that under basal conditions the GAP activity of AS160 could maintain a Rab protein in an inactive GDP-bound state. However, insulin could trigger signaling through Akt/PKB for AS160 phosphorylation and inhibition of its GAP domain, converting the Rab protein to its active GTP-bound form, thereby allowing GLUT4 translocation (50).

GLUCOSE TRANSPORT

Evidence suggesting that insulin increased glucose uptake by increasing the translocation of glucose transporters from an intracellular pool to the plasma membrane was first demonstrated in adipocytes (15). Shortly after, the results in adipocytes were confirmed in isolated diaphragm muscles (49) and then in rodent skeletal muscle (31). Utilizing the hindlimb perfusion technique, Klip et al. (31) found that insulin increased the number of plasma membrane associated glucose transporters 2-fold, while the intracellular glucose transporter concentration was reduced by 70%. Furthermore, the strong relationship between the number of GLUT4 transporters translocated to the plasma membrane and glucose transport was demonstrated using a labeled cell surface GLUT4 protein (20). Collectively, these studies suggested that insulin increases glucose uptake by increasing the number of plasma membrane associated glucose transporters.

Because the lipid bilayer of the plasma membrane is impermeable to glucose, a glucose transport system is required to move glucose by facilitated diffusion down its

concentration gradient from the extracellular to intracellular space. There are twelve homologous transmembrane glucose transport proteins (GLUT1-GLUT12) that have been identified (53). Each of which displays characteristically substrate specificity, kinetic properties and tissue distribution. For example, skeletal muscle expresses both GLUT1 and GLUT4. GLUT1, a ubiquitous glucose transporter, allows a constant influx of very low amounts of glucose under basal, or non-insulin-stimulated conditions. In contrast, in the absence of insulin GLUT4 is stored within the cytosol by specialized recycling endosomes known as vesicular tubular clusters (11, 50). However, in the presence of insulin GLUT4 is translocated from these intracellular pools to the plasma membrane.

Insulin-stimulation results in the remodeling of the actin cytoskeleton in which GLUT4 can be translocated from the cytosol to the plasma membrane. This process appears to be mediated by both the classical, or PI 3-kinae, and novel, or c-Cbl associated protein (CAP)/c-Cbl insulin signaling pathways. The convergence between these two pathways occurs at aPKC. aPKC activates its downstream target TC10, which is a distal components of the novel cascade. It has been demonstrated that TC10 is associated with Exo 70, an exocyst complex, which translocates to the plasma membrane following TC10 activation (24). Because overexpressing a mutated form of Exo 70 attenuated insulin-stimulated glucose uptake with no affect on GLUT4 translocation it was suggested that the exocyst complex may direct GLUT4 to the target-SNARE receptor (t-SNARE) complexes for docking and binding at the plasma membrane. Both cytochalasin D and Latrunculin B, pharmaceuticals that alter the actin cytoskeleton, inhibit insulin-stimulated glucose uptake and GLUT4 translocation, possibly by inactivating TC10 (24). The translocation of GLUT4 is therefore mediated by the cellular actin filaments. In addition, the Rho family GTPase Cdc42 are also thought to mediate actin remodeling. Cdc42

binds and activates the Wiskott-Aldrich Syndrome Protein (WASP). It has been suggested that WASP plays a role in altering the actin cytoskeleton as patients with WASP mutations exhibit defects in cell morphology (24). In addition, microtublule motor proteins kinesin KIF5b and KIF3 have been shown to facilitate GLUT4 translocation to the plasma membrane. Thus, it is possible that molecular motors move GLUT4 along tracks of actin, which under go a significant remodeling process under insulin-stimulated conditions (11).

In the presence of insulin GLUT4 exocytosis is increased while endocytosis is decreased. GLUT4 is recycled between vesicles and the cell surface via endosomal compartments (32) and localized to the trans-golgi network, clathrin-coated vesicles, and endosomes of the recycling pathway. Insulin-stimulated GLUT4 exocytosis is described by the SNARE model of GLUT4 trafficking in which GLUT4 is localized to tubulovesicular compartments enriched with the vesicle-SNAP receptor (v-SNARE) and the vesicle associated membrane protein-2 (VAMP2). The v-SNARE proteins VAMP2 and VAMP3/cellubrevin encompass part of the GLUT4 containing vesicles in skeletal muscle (11). Contact between GLUT4 containing vesicles and the plasma membrane is facilitated by the v-SNARE synaptobrevin and the target-SNAP receptor (t-SNAREs) syntaxin-4. According to this model, GLUT4 containing vesicles fuse with the plasma membrane via the complex interaction between VAMP2 with the t-SNARE complex which is composed of syntaxin 4 and the soluble NSF attachment protein-23 (SNAP23). It is the SNAP proteins then that regulate the association between the v-SNAREs and t-SNAREs (50).

However, tubulovesicular elements in the cytoplasm appear to contain the majority of GLUT4 (32). Thus, following insulin-stimulated translocation, GLUT4 accumulates in clathrin-coated portions of the plasma membrane via the AP2 adaptor

complex, which facilitates internalization through clathrin mediated endocytosis (32). The GTPase dynamin is a protein involved in GLUT4 endocytosis. Upon insulin stimulation dynamin translocates to the plasma membrane where it interacts with Grb2. The dynamin-Grb2 complex increases the GTPase activity of dynamin for the formation of clathrin-coated vesicles. Clathrin-coated GLUT4 containing vesicles are then moved through the endosomal recycling system where they loose their clathrin coat and can then be stored in specialized perinuclear reticular compartments (32).

THE MAMMALIAN TARGET OF RAPAMYCIN SIGNALING PATHWAY

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase and a member of the PI 3-kinase related family (21). The mTOR signaling pathway is activated by insulin, amino acids, growth factors and impaired by deficient nutrients and energy (48). The activity of mTOR is regulated by multiple phosphorylation sites. As suchm, ser-2481 is a site for autophosphorylation whereas ser-2448 and thr-2446, when phosphorylated, increase the activity of mTOR toward its downstream targets (21).

The best know inhibitor of mTOR is rapamycin. Rapamycin inhibits mTOR through FKBP12. Specifically, the rapamycin-FKBP12 complex inhibits the activity of the complex, raptor-mTOR-GβL, which regulates the kinase activity of mTOR (39). In the rapamycin insensitive complex raptor is replaced by rictor to form the rictor-mTOR complex (39). Thus, the primary binding partners of mTOR are raptor and rictor. These complexes are also referred to as mTORC1 and mTORC2 (48).

Insulin activates mTOR signaling, possibly through PI 3-kinase. Insulin activates PI 3-kinase, leading to the phosphorylation and activation of Akt/PKB. Akt/PKB in turn phosphorylates tuberous sclerosis complex 2 (TSC2), a small G-protein Rheb. The GAP activity is inhibited by phosphorylated TSC2, allowing Rheb to accumulate in its active

GTP-bound form (48). The accumulation of GTP-Rheb stimulates the kinase activity of mTOR. Furthermore, mTOR-RICTOR has been shown to phosphorylate Akt/PKB at Ser-473 in response to insulin. In contrast, the rapamycin sensitive mTOR/RAPTOR complex is a downstream target of Akt/PKB, placing mTOR both upstream and downstream of Akt function depending on its associated interacting partner.

mTOR signaling is also mediated by amino acids, specifically leucine. The precise mechanism of leucine induced activation of mTOR is unclear, but it does appear that it may be independent of TSC2. Amino acid withdrawal was found to inhibit mTOR signaling in TSC2-deficient cells (42). Recent evidence suggests that the TSC2 independent signal may act through the PI 3-kinase related protein HVPS34 (10). However, more work is needed to determine the involvement HVPS34, as well as additional PI 3-kinase related proteins, and their exact interaction with mTOR.

AMINO ACIDS

Amino acids are compounds containing both an amine and carboxylic group and a side chain that is unique to each amino acid. In general, amino acids are central to life and have many functions including their roles as the building blocks for proteins and as intermediates for metabolism. Of the 22 amino acids, 8 are classified as essential amino acids because the human body cannot synthesize them from other compounds. Therefore, the essential amino acids must be obtained from one's diet. The essential amino acids can further be divided into those exhibiting side chains with a branch and those with no branching. Amino acids having an aliphatic side-chain with a branch are termed branched-chain amino acids and include leucine, isoleucine and valine. The branched-chain amino acids are of particular importance because they account for a large

proportion of the essential amino acids in muscle protein, used clinically for a variety of aliments and recently, been shown to impact glucose metabolism.

Much work has been dedicated to the individual effects of amino acids. As a result of this research, it has become possible to combine amino acids in order to create mixtures that can have a far greater affect on, for example, glucose metabolism, than an individual amino acid can. Some amino acids are reported to have either a direct affect on glucose uptake or act indirectly through increases in insulin or muscle glycogen levels. Leucine (36), isoleucine (17, 18) and cysteine (23) have been shown to increase skeletal muscle glucose uptake in the absence of insulin. Although leucine increases insulin secretion, it is noteworthy that the elevated insulin levels do no account for its positive impact on glucose uptake. In addition, methionine also increases insulin secretion (12) but its exact role in glucose metabolism is unclear. Furthermore, both leucine (36) and valine have been shown to increase skeletal muscle glycogen synthesis (17)

Since amino acids must enter muscle cells before exerting their actions they require transport proteins, or systems, to move across the plasma membrane. There are several different transport systems located on the sarcolemma, each classified by the type of amino acid it transports, its transport mechanism and its regulation. The majority of skeletal muscle amino acid transport occurs through either System A or System L transporters. System A consists of secondary active transport that moves short, neutral amino acids across the plasma membrane against their concentration gradient that is both pH and insulin sensitive (13). In contrast, System L transports bulky, neutral amino acids as well as the branched-chain amino acids and is not regulated by pH or insulin (13). Because System L acts independent of sodium it uses non-System L amino acids to move its substrates across the plasma membrane. Thus, for each System L amino acid

that is transported into the muscle cell, a non-System L amino acid is transporter out of the cell (34).

EFFECTS OF BRANCHED-CHAIN AMINO ACIDS ON GLUCOSE TRANSPORT

In addition to insulin, branched-chain amino acids, specifically isoleucine and leucine may increase glucose uptake. It is well documented that branched-chain amino acids, especially leucine, are integral to protein synthesis, and this process appears to occur through mTOR (2). But recent studies suggest that both isoleucine and leucine may be involved in insulin signaling and glucose homeostasis. Nishitani et al. (36) investigated the effects of leucine on skeletal muscle glucose uptake in the absence of insulin. Using the isolated muscle preparation, soleus muscle incubated in leucine showed a significant increase in glucose uptake. Interestingly, the increase in glucose uptake for muscle bathed in leucine showed a 1.5 fold increase in glucose uptake, which was similar to that achieved by a physiological amount of insulin (36). However, the effect of leucine was suppressed by the administration of both LY294002, a specific inhibitor of PI 3-kinase and GF109203X, a specific inhibitor of PKC. In contrast, glucose uptake was not impaired when the isolated soleus was exposed to rapamycin, a specific inhibitor of mTOR, and leucine. These findings suggest that leucine may stimulate glucose transport via PI 3-kinase and PKC, and not mTOR and this appears to be independent of insulin.

Recent evidence suggests that isoleucine, rather than leucine is a stronger effector of glucose uptake and whole-body glucose homeostasis. An oral gavage of isoleucine decreased blood glucose levels and increased skeletal muscle glucose uptake (18). This occurred despite no significant increase in the circulating insulin concentration. Although the exact mechanism was not determined in this study, the authors did find that

hepatic glucose production was reduced in vitro (18). In addition, 4-hydroxyisoleucine (4-OH-ILE), an amino acid extract, has been shown to stimulate insulin secretion and improve glucose tolerance in diabetic rats (7, 8). The improved glucose tolerance may have resulted from increased glucose uptake and/or decreased hepatic glucose production. The authors also found that 4-OH-ILE may affect insulin signaling, as PI 3-kinase activity was increased (7). Collectively, these findings suggest that the branched-chain amino acids, specifically isoleucine and leucine may enhance glucose uptake and regulate glucose homeostasis.

COMBINED EFFECTS OF CARBOHYDRATES AND AMINO ACIDS ON GLUCOSE TRANSPORT

Although the individual affects of carbohydrates and specific amino acids have been demonstrated on glucose tolerance the combined effects are not as well defined. The individual administration of carbohydrate and leucine have been shown to enhance PI 3-kinase activity (6). But leucine has not always been shown to have a positive affect on PI 3-kinase activity. Baum et al. (5) compared the effects of an oral dose of carbohydrate, leucine or a combined carbohydrate and leucine supplement on glucose homeostasis and PI 3-kinase activity. Fifteen minutes post supplementation there was no difference in PI 3-kinase activity between carbohydrate and carbohydrate plus leucine. However, from 30-90 min post supplementation PI 3-kinase activity was higher in rats that consumed carbohydrate compared to the combined treatment. Despite reduced PI 3-kinase activity there was no difference in glucose uptake between carbohydrate and carbohydrate plus leucine treated rats. According to the study authors the initial activation of PI 3-kinase was sufficient to initiate glucose uptake (5). It is therefore possible that leucine and insulin enhance glucose transport via different mechanisms. As

previously stated, leucine may stimulate glucose transport by an unidentified process that is independent of the mTOR/PI 3-kinase pathway (36, 43).

Recent studies have demonstrated that a mixture of branched-chain amino acids may decrease blood glucose levels. Oral administration of a branched-chain amino acids mixture has been shown to reduce hyperglycemia in a virus-induced diabetic mouse model (44) and reduce blood glucose levels in streptozotocin-induced rats (19). However, it was not clear as to whether it was the mixture of branched-chain amino acids or an individual amino acid that improved glucose metabolism. In light of these findings, Doi et al. (17) investigated the individual effect of leucine, isoleucine and valine on blood glucose levels in rats and glucose uptake in cultured C_2C_{12} myotubes. Rats were gavaged with either leucine, isoleucine or valine 30 min prior an OGTT. The rats were then gavaged with a glucose solution and the blood glucose levels were traced over a 120 min time period. Isoleucine significantly reduced blood glucose levels at 30 min and 60 min post glucose administration. Leucine nor valine appeared to improve glucose tolerance under these conditions. In fact, valine increased blood glucose levels, possibly because valine, a glucogenic amino acid, increased gluconeogenesis. In C_2C_{12} myotubes, isoleucine had the greatest affect on glucose uptake, and its effect was independent of insulin. Using specific inhibitors for the insulin signaling cascade the authors found that the increased glucose uptake by isoleucine involved both PI 3-kinase and PKC. However, these improvements did not appear to involve mTOR as rapamycin enhanced glucose uptake in isoleucine treated myotubes.

GLUCOSE DISPOSAL

Following the transport of glucose across the plasma membrane and into the muscle cell, it is either converted to glucose-6-phosphate (G6P) by hexokinase or

counter-transporter back into the blood. At any given time this is largely controlled by the activity level of hexokinase and the glucose concentration gradient. After glucose is phosphorylated, it has two distinct fates; used as an immediate energy source via glycolysis or be converted to glycogen, a stored form of energy. Therefore, once phosphorylated by hexokinase, becoming G6P, the glucose molecule can not be transported back out of the cell as skeletal muscle lacks glucose-6-phosphatase. Under fasting conditions the majority of blood glucose following the ingestion of a carbohydrate load will be removed by skeletal muscle and the primary fate of the cleared glucose will be its conversion to glycogen. After the formation of G6P, it is transformed to glucose-1-phosphate (G1P) by the enzyme phosphoglucomutase. G1P and uridine triphosphate (UTP) is then synthesized to uridine diphosphate (UDP)-glucose in a reaction catalyzed by UDP-glucsoe pyrophosphorylase. The C-1 carbon of UDP-glucose is considered "activated" because its hydroxyl group is esterfied to the diphosphate moiety of UDP. New glucose units can now be added to the non-reducing end of glycogen. The UDP-glucose is transferred to the hydroxyl group at a C-4 terminus of glycogen forming an α-1-,4 glycosidic linkage. While the glycogen macromolecule becomes longer its terminal hydroxyl group displaces UDP. Glycogen synthase (GS) is the enzyme for this elongation reaction. However, GS only catalyzes α-1,4-linkages, thereby synthesizing a linear macromolecule. In order for glycogen to increase its storage capacity, solubility and the number of non-reducing ends, a branched polymer is needed. Thus, a branching enzyme breaks the α-1,4 linkages and forming α-1,6-linkages to form branches.

The rate limiting enzyme for glycogen synthesis is GS (33). It is regulated by multiple phosphorylation sites and by several allosteric effectors (38, 47). Of these potential phosphoryaltion sites, ser-7 (site 2), ser-10 (site 2a), ser-640 (site 3a), ser-644 (site 3b) appear to be the most important regulating residues on GS (41). In general,

phoshporylation reduces the catalytic activity of GS, causing an increase in the Km for the substrate UDP-glucose and an increase in the Ka for G6P (47). Although there are several protein kinases that phosphorylate GS, glycogen synthase kinase-3 (GSK-3) appears to have the strongest inhibitory effect (41). However, the amount of inactivation depends not only on the sites phosphorylated but the concentration of allosteric effectors such as G6P.

The molecule G6P is a potent allosteric activator of GS. Despite GS being phosphorylated at multiple sites, G6P can still fully activate GS (47). It is believed that G6P activates GS via dephosphoryaltion of the enzyme (38). The effect of G6P is enhanced by the presence of insulin. Therefore, dephosphorylation of GS by insulin decreases the concentration of G6P needed to activate the enzyme (46).

In skeletal muscle, GS exists in either the I-form or D-form. The I-form is active in the absence of G6P. Whereas the D-form is active in the presence of G6P but its activity is attenuated when G6P is absent. When at least three sites of GS are phosphorylated, the enzyme is quickly converts back to its less active D-form (25).

INSULIN RESISTANCE

Insulin resistance is the condition that occurs when a normal level of insulin produces a less than normal biological response (27). As a result, excess insulin must be secreted in order to achieve a normal blood glucose response. Insulin resistance can be further characterized by its level of insulin sensitivity and/or insulin responsiveness. Insulin sensitivity is defined by the percent of a maximal biological response, or glucose transport, caused by a submaximal insulin concentration. Therefore, a higher percent of a maximal biological response at a given submaximal insulin concentration represents an increased insulin sensitivity (27). In contrast, insulin responsiveness is defined by the

maximal biological response, or glucose transport, caused by a maximal insulin concentration (27). Thus, a higher maximal biological response indicates greater insulin responsiveness.

Being able to distinguish between insulin sensitivity and responsiveness is critical to identifying potential locale(s) of insulin resistance. A reduction in insulin sensitivity indicates potential impairments to the insulin receptor and/or signals leading to the receptor. For example, reduced insulin sensitivity could be caused by a reduction in the number of insulin receptors or by reduced affinity of the receptor towards insulin (27). In contrast, reduced insulin responsiveness indicates a post insulin receptor defect such as impaired glucose transport and/or glucose disposal (27).

THE ZUCKER RAT

Zucker rats are the most commonly used rodent model for obesity and insulin resistance research. The obese Zucker rat exhibits severe insulin resistance due to both decreased insulin sensitivity and insulin responsiveness (16, 40). The genetically obese Zucker rat was first described in 1961 by Zucker and Zucker (54). This rodent model of insulin resistance was the result of a single recessive gene when Merck Stock M and Sherman rats were cross bred (3). The symbol fa/fa is used to denote the obese condition, whereas the lean litter mates are symbolized by fa/?. Upon visual inspection, from the time of birth and up to 4 weeks of age homozygous rats are not easily distinguishable from their heterozygous litter mates. However, beyond 4 weeks of age the obese Zuckers appear "fatter" compared to their lean litter mates as easily seen by their noticeable rounder appearance (3).

The obese Zucker rat is characterized by insulin resistance, hyperinsulinemia and hypertriglyeridemia (3), which is due largely to their rapid onset of obesity. Both

hyperphagia and decreased activity levels are major factors in the Zucker rat becoming obese (3, 45). The hyperphagia is primarily due to a defective leptin receptor (37). Leptin is a molecule that binds to specific receptors in the hypothalamus and controls satiety. Under normal conditions, such as in the lean Zucker rat leptin will bind to its receptor causing the rat to feel full and stop eating. In contrast, obese Zucker rats have defective leptin receptors which prevent leptin from successively binding. Since leptin can not bind to its receptor the rat continuously eats because it does not feel full. The excess caloric intake leads to the rapid development of obesity and insulin resistance. The obesity, or excess fat mass, in obese Zucker rats is due to an increase in both adipose cell size and number (3). The significantly larger adipose cell size can be partially explained by increased levels of lipoprotein lipase, an enzyme that stores lipids (45).

As previously stated, the obese Zucker rat has impaired insulin sensitivity and insulin responsiveness. Insulin must only bind to approximately 20% of the insulin receptors to elicit a maximal insulin response. Under physiological conditions, the number of insulin receptors on the cell membrane will not decline by more than 50-60% below normal, suggesting that decreased insulin responsiveness is due to a post receptor defect. Thus, the reduced insulin responsiveness in obese Zucker rats appears to lie in the glucose transport process (20, 26, 40). While the reduced insulin sensitivity observed in obese Zucker rats is due to decreased insulin receptor number (14).

Surprisingly, skeletal muscle GLUT4 expression of obese Zucker rats does not appear to be reduced (20, 22). Rather, the insulin-stimulated translocation of GLUT4 to the plasma membrane is impaired (20, 30). Sherman et al. (40) reported that the rates of insulin-stimulated glucose transport were significantly reduced in obese compared to lean Zucker rats. Although not directly measured in this study, the total cellular concentration of GLUT4 could not account for the decline in insulin-stimulated glucose transport (4).

A follow up study conducted by Brozinick et al. (9) investigated the translocation of GLUT4 from its intracellular compartments to the plasma membrane. Upon insulin-stimulation in the lean rats there was an increase in GLUT4 at the plasma membrane with a parallel decrease of the intracellular GLUT4. However, insulin stimulation did not increase the number of plasma membrane associated glucose transporters in the obese rats suggesting a defective GLUT4 translocation process in the skeletal muscle of obese Zucker rats. This finding was later confirmed by Etgen et al. (20) using ATB-BMPA, a precise labeling method for plasma membrane GLUT4.

It is widely accepted that the defect in skeletal muscle GLUT4 translocation observed in obese Zucker rats can be attributed to impairments in insulin signaling. Using the hind limb perfusion technique Anai et al. (1) reported that obese Zucker rats had reduced IRS-1 protein concentration, IRS-1 tyrosine phosphorylation and IRS-1 associated PI 3-kinase activity under insulin-stimulated conditions compared to their lean litter mates. Thus, there are marked defects in specific insulin signaling proteins and GLUT4 translocation that characterize the skeletal muscle insulin resistance of obese Zucker rats.

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Chapter III: An amino acid mixture improves glucose tolerance and insulin signaling in Sprague Dawley rats

ABSTRACT

The first aim of this investigation (experiment [Exp]-1) was to evaluate the effect of an amino acid supplement on the glucose response to an oral glucose challenge. The second aim of this investigation (experiment [Exp]-2) was to evaluate if differences in blood glucose response were associated with increased skeletal muscle glucose uptake. Exp-1 rats were gavaged with either glucose (CHO), glucose plus an amino acid mixture (CHO-AA-1), glucose plus an amino acid mixture with increased leucine concentration (CHO-AA-2) or water (PLA). CHO-AA-1 and CHO-AA-2 had reduced similar blood glucose responses compared to CHO with no difference in insulin among the four treatments. Rats in Exp-2 were gavaged with either CHO or CHO-AA-1. Fifteen min after gavage a bolus containing [^{3}H] 2-deoxyglucose and [U-^{14}C] mannitol was infused via a tail vein. Blood glucose was significantly lower in CHO-AA-1 than CHO, while insulin responses were similar. Muscle glucose uptake was higher in CHO-AA-1 compared with CHO in both fast-twitch red (8.36 $\pm$ 1.3 vs 5.27 $\pm$ 0.7 μmol/g/h) and white muscle (1.85 $\pm$ 0.3 vs 1.11 $\pm$ 0.2 μmol/g/h). There was no difference in Akt/PKB phosphorylation between treatment groups, however, the amino acid treatment resulted in increased AS160 phosphorylation in both muscle fiber types. Glycogen synthase phosphorylation was reduced in fast-twitch red muscle of CHO-AA-1 compared to CHO, while mTOR phosphorylation was increased. Phosphorylation of glycogen synthase and mTOR in fast-twitch white muscle did not differ between treatments. These findings suggest that an amino acid supplement improves glucose tolerance and that these

improvements are associated with an increase in skeletal muscle glucose uptake possibly due to improved intracellular signaling.

INTRODUCTION

It is well documented that amino acids, specifically the branched-chain amino acids, are essential for protein synthesis. Emerging literature, however, suggests that amino acids may also play a role in glucose homeostasis. Of the branched-chain amino acids, isoleucine and leucine appear to be the primary amino acids affecting blood glucose levels. Both of these branched-chain amino acids have demonstrated glucose lowering abilities (4, 6, 22). Yet there remain many questions as to how amino acids affect blood glucose levels. Possible mechanisms that have been previously explored include a greater insulin response and/or an increased rate of skeletal muscle glucose clearance.

Insulin is a potent stimulator of skeletal muscle glucose transport. It has been shown that the combined ingestion of carbohydrates with either protein (24, 39) or amino acids (11, 25, 35, 36) may synergistically enhance insulin secretion. The glucose lowering effect of these compounds, therefore, has generally been assumed to be the result of a greater insulin response (8, 36). However, separate studies utilizing *in vitro* models have reported that leucine and/or isoleucine enhanced skeletal muscle glucose uptake under insulin-free conditions (4, 22), suggesting that insulin alone may not explain protein and/or amino acid-induced glucose clearance. Rather, factors beyond an increase in plasma insulin, such as altered muscle cell signaling, could be associated with this improved glucose tolerance.

As previously mentioned, both leucine and isoleucine have been found to increase skeletal muscle glucose uptake in the absence of insulin (4, 6, 22). Nishitani et al. (22)

reported that leucine-induced glucose uptake in isolated soleus muscle was negated when incubated with specific inhibitors for either phosphatidylinositol 3-kinase (PI 3-kinase) or atypical protein kinase C (aPKC). In contrast, pre-treatment with rapamycin, a specific inhibitor of the mammalian target of rapamycin (mTOR) had no effect. In agreement, Doi et al. (4) reported that isoleucine-induced glucose uptake in C_2C_{12} myotubes was inhibited in the presence of a specific inhibitor of PI 3-kinase but not in the presence of an inhibitor of mTOR.

Although *in vitro* results support the involvement of PI 3-kinase in amino acid-induced muscle glucose uptake under insulin-free conditions, this effect in combination with insulin and on blood glucose clearance *in vivo* is unclear. Therefore, the primary aim of this investigation (experiment [Exp]-1) was to determine if an amino acid mixture improves glucose tolerance in Sprague Dawley rats, and if differences in glucose tolerance are due to differences in skeletal muscle glucose uptake (experiment [Exp]-2).

MATERIALS AND METHODS

Animal care and housing. Exp-1: Twenty-four male Sprague Dawley rats approximately 10 weeks old were obtained from Harlan (Indianapolis, IN). Rats were randomly assigned to one of four groups: carbohydrate (CHO, n = 6), carbohydrate plus a 5-amino acid mixture (CHO-AA-1, n = 6), carbohydrate plus a 5-amino acid mixture with increased leucine concentration (CHO-AA-2, n = 6) or placebo (PLA, n = 6). Rats were housed individually and provided standard laboratory chow and water ad libitum. The temperature of the animal room was maintained at 21° C, with an artificial 12:12 h light-dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conformed

to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Exp-2: Twelve male Sprague Dawley rats approximately 12 weeks old were obtained from the University of Texas at Austin Animal Resources Center. The rats were then randomly assigned to either carbohydrate (CHO, n = 6) or carbohydrate plus a 5-amino acid mixture (CHO-AA-1, n = 6). Rats were housed individually and provided standard laboratory chow and water ad libitum. The temperature of the animal room was maintained at 21° C and an artificial 12:12 h light-dark cycle was set. All experimental procedures were approved by the IACUC of the University of Texas at Austin and conformed to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Experimental protocol. Exp-1: Following 1 week of acclimation, rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior to the testing to familiarize them with the experimental procedures. After a 12 h fast, each rat was wrapped in a towel and the tip of their tail cut and bled. Rats were orally gavaged (8 ml/kg body weight) with 1 of 4 solutions: 1) CHO (22.5% glucose), 2) CHO-AA-1 (amino acid mixture in 22.5% glucose), 3) CHO-AA-2 (amino acid mixture with increased leucine concentration in 22.5% glucose) or 4) PLA (distilled water). The amino acid mixture contained 5.28 mg cysteine, 3.36 mg methionine, 6.68 mg valine, 944.8 mg isoleucine and 6.68 mg leucine per 50 ml solution. The leucine concentration was increased to 50 mg/50 ml for the CHO-AA-2 treatment. Blood (0.5 ml) was taken from the tail immediately before the gavage and 15, 30, 60 and 120 min after. Blood was collected in one test tube containing EDTA (24 mg/ml, pH 7.4), and 0.1 ml of the EDTA blood sample was transferred to another test tube containing 10% perchloric acid (PCA). These tubes were stored at -80° C for later analysis. Immediately after the 120 min blood

sample, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75.0 mg/kg body weight) at which time the red and white gastrocnemius were excised, freeze clamped in liquid nitrogen and stored at -80° C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital.

Exp-2: Following 1 week of acclimation, rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior to the testing to familiarize them with the experimental procedures. After a 12 h fast the rats were wrapped in a towel and the tip of their tail cut for blood sampling as described above. The rats were then orally gavaged (8 ml/kg body weight) with either CHO (22.5% glucose) or CHO-AA-1 (amino acid mixture in 22.5% glucose). The amino acid mixture was described previously in Exp-1. Fifteen min after the gavage, a bolus containing 40 μCi/kg body weight [^{3}H] 2-deoxyglucose (2-DG) and 20 μCi/kg body weight [U-^{14}C] mannitol was injected by syringe via a tail vein. Blood samples were collected before gavage, and at 2, 10, 20 and 45 min after the radioactive tracers were infused. Immediately after the 45 min blood sample, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75.0 mg/kg body weight) at which time the red and white gastrocnemius were excised, freeze clamped with tongs cooled in liquid nitrogen and stored at -80° C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital.

Blood analysis. A drop of blood was used to measure blood glucose with a portable glucose analyzer (One Touch Ultra 2; LifeScan Inc., Milpitas, CA). Before using the glucose analyzer several blood samples were measured using the Trinder assay (34) to verify its validity and reliability. The glucose analyzer was calibrated according to the manufacturer's instructions. EDTA samples were then centrifuged 14,000 x g for

10 min at 4° C. Plasma insulin (Linco, cat. # SRI-13K, St. Charles, MO) was determined by a radioimmunoassay kit according to the manufacturer's instructions.

Muscle glucose uptake. Rates of 2-DG uptake were determined in both red and white gastrocnemius muscle samples. Approximately 80-100 mg of muscle were dissolved in 1 N potassium hydroxide (KOH) by incubating for 15 min at 65° C, vortexed then incubated for an additional 5 min at 65° C. Next, an equal volume of 1 N hydrochloric acid (HCl) was added to the digested samples and vortexed to neutralize the samples. To determine the specific activity of the blood, an aliquot of the PCA extract was added to 1 N KOH, then neutralized with an equal volume of 1 N HCL. A 300 µl aliquot of neutralized muscle and blood samples were then added to a vial containing 6 ml Bio-Safe II counting cocktail (Research Products International, Mount Prospect, IL). Duplicate samples were counted in a liquid scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA) preset for simultaneous counting of [^{3}H] and [^{14}C] DPM. Quenching was determined by counting prepared standards. The accumulation of intracellular 2-DG is indicative of muscle glucose uptake. The specific activity of the blood for [^{3}H] and [^{14}C] was determined using the integral of the plasma 2-DG and [U-^{14}C] mannitol over the 15-60 min per glucose molecule. The extracellular space was calculated using the total muscle ^{14}C DPM and its specific activity. The intramuscular accumulation of 2-DG was calculated by subtracting its extracellular space DPM from its total muscle DPM divided by its specific activity.

Tissue Processing. Approximately 60 mg of muscle was homogenized (1:9) in an ice-cold buffer (pH 7.4) containing 20 mM HEPES, 2 mM EGTA, 50 mM sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerolphosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM Benzamidine, and 0.5 mM Na Vanadate (1 ml/100 mg muscle) with a glass tissue grinder pestle (Corning Life Sciences,

Acton, MA). The homogenate was then centrifuged at 14,000 x g for 10 min at 4° C. Aliquots of the supernatant were stored at -80° C for later analysis. The protein concentration of the homogenate was determined using the Lowry method (18).

Tissue analysis. The phosphorylation of protein kinase B (Akt/PKB), mTOR, Akt substrate of 160 kDa (AS160) and glycogen synthase (GS) were used as an indirect measurement of activity. Sample protein (100 μg for Akt/PKB, 70 μg mTOR and AS160 and 60 μg for GS) was combined with an equal amount (1:1) of Laemmli sample buffer (125mM tris, 20% glycerol, 2% SDS, 0.008% bromophenol blue, pH 6.8) (16) and boiled for 5 min. Next, sample proteins were subjected to SDS-PAGE and the proteins separated on either an 8% (mTOR, AS160) or 12% (Akt/PKB and GS) polyacrylamide resolving gel for either 1 h (Akt/PKB) or 1.5 h (mTOR, AS160 and GS). The resolved proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer unit and blocked in 7% nonfat dry milk in Tris-Tween-buffered saline (NFDM/TTTBS) for 1 h at room temperature (RT). The membranes were then incubated with either affinity purified anti-phospho-Akt/PKB (Thr-308) (Cell Signaling Technology, Danvers, MA), anti-phospho-mTOR (Ser-2448) (Cell Signaling Technology), anti-phospho-AS160 (Thr-642) (Millipore, Billerica, MA) or anti-phospho-GS (Ser-641) (Cell Signaling Technology) overnight at 4° C. These phosphorylation sites were chosen as an indirect measurement of activity because they represent the primary sites of phosphorylation of the respective protein under insulin-stimulated conditions. The primary antibodies were diluted to either 1:500 (phospho-AKT/PKB), 1:800 (phospho-AS160) or 1:1000 (phospho-mTOR, phospho-GS) in TTBS containing 1% NFDM. Following the overnight incubation the membranes were washed for 3, 5-min washes in TTBS then incubated for either 1 h (Akt/PKB and GS) or 2 h (mTOR, AS160) at RT with the species-specific (anti-rabbit) immunoglobulin G (IgG) secondary

antibodies (Cell Signaling Technology). The secondary antibodies were diluted to either 1:750 (Akt/PKB, AS160), 1:900 (mTOR) or 1;2000 (GS) in TTBS containing 1% NFDM. The membranes were washed with 5, 8-min washes with TTBS and antibody binding was visualized by enhanced chemiluminescence in accordance to the manufacturer's instructions (Perkin Elmer, Boston, MA). Images were captured by using a charge-coupled device camera in a ChemiDoc system (BioRad, Hercules, CA) and saved to a computer. Density of the bands were quantified with Quantity One analysis software (BioRad) and expressed as a percentage of a standard run on each gel.

After the phosphorylation status of each protein had been determined, the primary phosphorylated-antibody was stripped from the membrane to determine total protein concentration. Membranes were placed in a stripping solution containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris base (pH 6.7) and heated at 60° C for 1 h. Membranes were washed for 3, 15-min washes with TTBS to remove the stripping solution. The primary and secondary antibody concentrations, incubation times, washing and quantification were the same as those described for the determination of phosphorylation status.

Statistical analysis. A two-way ANOVA was performed on the blood data (treatment x time). A one-way ANOVA was performed for the muscle tissue analysis. When a significant F-ratio was obtained, a Fisher's Least Significant Difference post hoc test was performed to identify statistically significant differences ($P < 0.05$) between means. All statistical analyses were completed using SPSS software (SPSS Inc., Chicago, IL) and all values expressed as means $\pm$ standard error (SE).

RESULTS

Animal characteristics. For Exp-1, there were no significant differences in body mass among treatment groups (CHO 284.4 $\pm$ 7.4 g; CHO-AA-1 288 $\pm$ 3.2 g; CHO-AA-2 284 $\pm$ 4.2 g; PLA 286.8 $\pm$ 5.1 g) on the day of testing. Likewise, for Exp-2, the body mass of the CHO (365.8 $\pm$ 7.6 g) and CHO-AA-1 (366.3 $\pm$ 6.6 g) groups were similar on the day of testing.

OGTT glucose and insulin. For both experiments, glucose and insulin concentrations were determined following a 12 h fast and during the OGTT. For Exp-1, there were no significant differences in fasting glucose or insulin levels among treatment groups. There was little change in the blood glucose response for PLA throughout the OGTT. This would indicate no undue stress on the animals. Blood glucose was significantly lower for CHO-AA-1 and CHO-AA-2 compared to CHO at 15, 30 and 60 min post supplementation (Figure 3.1A). The glucose area under the curve (AUC) was also lower for CHO-AA-1 and CHO-AA-2 compared to CHO (Figure 3.1B). However, there were no differences in plasma insulin levels between CHO, CHO-AA-1, or CHO-AA-2 treatment groups at any time point (Figure 3.2A) or for the insulin AUC (Figure 3.2B). Both the blood glucose and plasma insulin concentrations were similar for CHO-AA-1 and CHO-AA-2 at all time points, and for the AUC as well, during Exp-1. Similar to the results of Exp-1, blood glucose levels were significantly reduced for CHO-AA-1 compared to CHO (Figure 3.3A) with no difference in plasma insulin response (Figure 3.4A) during Exp-2. The glucose AUC was lower for CHO-AA-1 compared to CHO (Figure 3.3B), but insulin AUC was similar between the two treatments (Figure 3.4B).

Muscle glucose uptake. Rates of skeletal muscle 2-DG uptake are shown in Figure 3.5. Glucose uptake in the red gastrocnemius was enhanced for the CHO-AA-1 compared to the CHO treatment group in Exp-2. A similar response was observed in the

white gastrocnemius as glucose uptake was greater for the CHO-AA-1 compared to the CHO.

Protein concentration and phosphorylaiton status of signaling proteins. The phosphorylation status of insulin signaling proteins for Exp-2 was assessed as an indirect measurement of their activity level. Akt/PKB protein concentration and Thr-308 phosphorylaiton were not different in both the red and white gastrocnemius from CHO and CHO-AA-1 treatment groups (Figures 3.6A-B). Although the protein concentration of the red and white gastrocnemius for mTOR did not differ between treatment groups, mTOR phosphorylaiton at Ser-2448 was significantly increased for CHO-AA-1 compared to CHO in the red, but not white muscle (Figures 3.7A-B). There was no difference in GS protein concentration between treatment groups. Phosphorylation of GS at Ser-641 was significantly lower in the red gastrocnemius of CHO-AA-1 compared to CHO (Figure 3.8A). This difference, however, between groups was not noted in the white gastrocnemius (Figure 3.8B). Finally, despite no difference in red and white gastrocnemius AS160 protein concentration, AS160 Thr-642 phosphorylation levels were significantly greater for CHO-AA-1 than in CHO across the fiber types (Figures 3.9A-B).

DISCUSSION

The present study found that the addition of an amino acid mixture to a carbohydrate supplement improved glucose tolerance in Sprague Dawley rats. Using an OGTT in Exp-1 we demonstrated that rats co-ingesting a carbohydrate and amino acid supplement, compared to receiving carbohydrate alone, lowered the blood glucose response to the glucose challenge. Since insulin is a potent stimulator of blood glucose clearance, we measured plasma insulin levels to help explain differences in the blood glucose responses. Although insulin secretion is mainly regulated by the blood glucose

level, protein and some amino acids stimulate pancreatic insulin secretion (7, 30, 35). Furthermore, the addition of protein or amino acids to a carbohydrate supplement has been found to result in a greater insulin response than when either is consumed alone (11, 24, 35, 36, 39). However, in the present study, the co-ingestion of carbohydrate plus an amino mixture did not result in a greater insulin response compared to carbohydrate consumed alone. This may be due to the relatively low amino acid concentration of our mixture, or possibly the rate of amino acid entry into the gut. Regardless, it is unlikely that the lower glucose response with the cabohydrate/amino acid mixture was due to greater insulin availability.

In an attempt to elicit a more pronounced effect of the amino acid mixture on plasma insulin and blood glucose in the current investigation, we increased the leucine concentration of the amino acid mixture. Leucine is known to stimulate insulin secretion *in vivo* (7). Moreover, leucine has been shown to increase glucose uptake in C_2C_{12} myotubes and in isolated soleus muscle (4, 22). Because these studies demonstrated amino acid-stimulated glucose uptake in the absence of insulin, we investigated the possibility that a further increase in leucine content within our amino acid mixture could initiate a greater blood glucose clearance *in vivo*. However, our results show that increasing the leucine concentration of the amino acid mixture from 0.13 mg/ml (CHO-AA-1) to 1 mg/ml (CHO-AA-2) had no further affect on either plasma insulin secretion or blood glucose response. Nishitani et al. (22) incubated isolated soleus muscle in medium with a leucine concentration between 0 – 4.0 mM. The authors reported that 2 mM leucine concentration was optimal for *in vitro* muscle glucose uptake, with minimal glucose uptake with leucine concentrations less than 2 mM and reduced uptake when leucine of more than 2 mM was added to the medium. The present investigation did not test a range of leucine concentrations *in vivo* in Exp-1. Rather, we assessed blood

glucose response to ingesting an amino acid mixture containing either 1.0 or 7.6 mM leucine. It is difficult to make direct comparisons between *in vitro* and *in vivo* results and when using a mixture of amino acids as opposed to assessing the effects of just one amino acid. However, it appears that increasing the concentration of leucine in our amino acid mixture had no additional affect on the blood glucose or insulin responses to a glucose challenge.

There are several possible explanations for the lower blood glucose response during the CHO-AA trial compared to the CHO trial. During an oral glucose tolerance test, the rate of glucose appearance is, in part, controlled by the rate of gastric emptying of the glucose, and in this regard, protein has been found to reduce the rate of gastric emptying (17). Therefore one possibility for the lower glucose response during the CHO-AA trials is a slowed rate of gastric emptying. However, considering the amount of protein required to limit gastric emptying, it is highly unlikely that the small amount of amino acids provided in our carbohydrate/amino acid supplement would have such an effect (19).

The second possibility for an improved glucose tolerance following ingestion of our amino acid mixture is that the amino acids suppressed hepatic glucose output thereby lowering the rate of glucose appearance. Doi et al. (6) demonstrated reduced phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) mRNA expression in isolated hepatocytes incubated with isoleucine under insulin-free conditions. The activity of G6Pase was also reduced under these conditions. This raises the possibility that reduced hepatic gluconeogenesis may partially explain why blood glucose was lower when our rats were treated with our amino acid mixtures. Conversely, insulin is a strong inhibitor of hepatic glycogenolysis and gluconeogenesis (28) with relatively small increases in blood insulin levels capable of completely suppressing

hepatic glucose output. In the present study, plasma insulin reached levels in excess of 350 pmol/L, and were not significantly different among treatments. These levels of insulin should completely suppress hepatic glucose output and negate any effect our amino acid mixtures might have had on limiting hepatic glucose output. Therefore, it is highly unlikely that the differences in blood glucose response for our CHO-AA and CHO trials were due to differences in hepatic glucose output.

A third possibility for the lower blood glucose response during the CHO-AA trials is an increase in peripheral glucose uptake. Skeletal muscle is the predominant peripheral tissue for blood glucose clearance (3, 12). In Exp-2, oral gavage of CHO-AA-1 produced greater skeletal muscle glucose uptake in both red and white skeletal muscle compared with the administration of CHO alone. Similar to what we observed in Exp-1, this effect did not appear to be the result of enhanced insulin secretion in Exp-2. Thus, these results indicate that amino acids can enhance insulin-stimulated muscle glucose uptake *in vivo* in the presence of insulin.

Several studies implicate the insulin signaling protein, PI 3-kinase, in amino acid-stimulated glucose uptake. Doi et al. (4) used the PI 3-kinase inhibitor wortmannin to demonstrate that isoleucine-stimulated glucose uptake in C_2C_{12} myotubes occurred in a PI 3-kinase dependent manner. Using specific inhibitors for both PI 3-kinase and aPKC, Nishitani et al. (22) reported that leucine stimulated glucose uptake required both PI 3-kinase and aPKC activation under insulin-free conditions *in vitro*. It was also reported by Morifuji et al. (20) that whey protein hydrolysates and dipeptides of branched-chain amino acids increased muscle glucose uptake and glycogen storage *in vivo* and that the increase in glycogen storage was directly related to the phosphorylation of downstream proteins of PI 3-kinase.

In the present investigation we assessed the phosphorylation status of Akt/PKB and AS160. Activation of PI 3-kinase results in the phosphorylation and activation of Akt/PKB, and possibly specific aPKCs. AS160 is a downstream substrate of Akt/PKB, and the phosphorylation of AS160 by Akt/PKB inhibits its activity. It has been proposed that an active GTP-bound Rab is needed for GLUT4 translocation, and that AS160 maintains a Rab protein in its inactive GDP-bound state under basal conditions. In the presence of insulin, AS160 is phosphorylated, allowing the conversion of the Rab protein to its activated GTP-bound form, enabling GLUT4 translocation (37). Interestingly, we did not find treatment differences in Akt/PKB protein concentration or its Thr-308 phosphorylation. Although insulin is a strong activator of Akt/PKB, this protein is not known to be activated by amino acids (9, 26). Our finding is in agreement with a previous report that an amino acid mixture improved glucose tolerance despite no increase in Akt/PKB phosphorylation in rats with liver cirrhosis (23).

Despite no difference in Akt/PKB phosphorylation between treatments, we did observe enhanced phosphorylation of AS160 in rats gavaged with CHO-AA-1 compared with rats gavaged with CHO only. Phosphorylation was elevated in both fast-twitch red and fast-twitch white muscle fibers. Since we did not observed a difference in insulin concentrations between trials, our findings suggest amino acid-stimulated glucose transport is associated with increased AS160 phosphorylation. It is then possible that a protein upstream of AS160, besides Akt/PKB, phosphorylates AS160 in the presence of amino acids. Akt/PKB, AMP-activated protein kinase (AMPK) and calcium signaling mechanisms have all been shown to activate AS160 (14. 31). However, only Akt/PKB has been implicated in insulin-stimulated glucose uptake, and Doi et al. (5) reported that amino acids increase glucose uptake without increases in AMPK activity. In light of no difference in Akt/PKB phosphorylation between the CHO-AA-1 and CHO treated rats,

we are unable to suggest a mechanism by which amino acids increase AS160 phosphorylation. However, the results of our study and others (9, 26) suggest that Akt/PKB is not involved and a link between amino acids and AS160 remains unclear.

Once glucose enters the cell it is phosphorylated by hexokinase to form glucose-6-phosphate and rapidly metabolized. The rapid removal of glucose-6-phosphate is essential, as an accumulation of glucose-6-phosphate would result in product inhibition of hexokinase, intracellular free glucose accumulation and its counter transport. In resting skeletal muscle, the majority of glucose-6-phosphate is converted to glycogen (38). The rate-limiting enzyme for glycogen synthesis is glycogen synthase, which is activated by its dephosphorylation.

We found that GS phosphorylation was reduced in fast-twitch red muscle of CHO-AA-1 treated rats compared with CHO treated rats. We did not find a significant difference in the phosphorylation of GS in fast-twitch white muscle between the CHO-AA-1 and CHO treated rats. Interestingly, the phosphorylation pattern of mTOR was inversely related to that of GS. That is, we observed a greater phosphorylation of mTOR in fast-twitch red muscle from CHO-AA-1 treated rats compared with CHO treated rats, but not in fast-twitch white muscle.

It is well established that an increase in amino acids, as well as insulin (13) can result in the phosphorylation of mTOR. Findings by Nishitani et al. (22) indicate that mTOR is not directly involved in amino acid-stimulated glucose transport. Similarly, Doi et al, (4) reported that isoleucine-stimulated glucose transport was independent of activation of mTOR. However, the ability of amino acids to activate mTOR in the presence of insulin (1) could have an additive effect with insulin in activating proteins involved in the intracellular disposal of glucose, if not transport. Activation of mTOR normally leads to the phosphorylation and subsequent activation of p70S6K, which in

turn can inactivate GSK-3, an upstream inhibitor of GS. Our observation that GS phosphorylation followed an inverse pattern to the phosphorylation of mTOR, therefore, suggests that the decreased in GS phosphorylation during the amino acid treatment occurred via the mTOR pathway. This agrees with earlier studies in which it was suggested that leucine can activate GS through the PI 3-kinase and mTOR pathways (2, 26). In addition, the combination of carbohydrate/protein supplementation post exercise is associated with an increase in muscle glycogen synthesis, the phosphorylation of mTOR and GSK-3 and the dephosphorylation of GS (10, 21, 38). It therefore appears that amino acid-stimulated glucose transport *in vivo* is tightly coupled with an increase in intracellular glucose disposal.

The present investigation suggests a role for amino acids in improved glucose tolerance. However, some studies report that amino acids may induce insulin resistance (15, 27, 29) and it is known that circulating amino acids are increased under insulin resistant conditions. Some potential mechanisms for amino acid-induced insulin resistance are decreased glucose transport due to reduced PI 3-kinase activity resulting from IRS-1 Ser/Thr phosphorylation by mTOR (15), or breakdown of the IRS-1 associated PI 3-kinase complex (33). Possible reasons for differences in results from the present investigation and those that report amino acids have no affect or reduce glucose tolerance, could be the methods used to administer the amino acids, the specific amino acids administered or the amount of amino acids consumed. For example, Krebs et al. (15) and Pisters et al. (27) reported that infusing amino acids in healthy adults significantly reduced whole body glucose disposal. These studies clamped insulin levels and continuously infused a 10% amino acid infusate, which is considerably more concentrated then the amino acid mixture that was gavaged in the present study. In a similar manner, Tremblay et al. (32) clamped the plasma essential amino acid

concentration at 6 mM for 4 h and observed a decrease in glucose tolerance. Furthermore, it was reported that concentrations of isoleucine (4) and leucine (22) up to 2 mM increased muscle glucose uptake *in vitro*, but that concentrations of 4 mM or greater had no effect. Considering all the results available, it would appear that the effects of amino acids on blood glucose regulation and muscle glucose uptake are amino acid-concentration dependent.

In conclusion, the present investigation demonstrated a lower blood glucose response to a glucose challenge when an amino acid mixture was provided simultaneously with the glucose. The lower glucose response appeared due to a greater rate of glucose clearance by skeletal muscle. This response did not appear to be due to a greater insulin secretion, but due to a greater activation of intracellular signaling proteins involved in glucose transport and intracellular glucose disposal. The long-term use of a carbohydrate and amino acid supplement, and whether it will be useful in the treatment of insulin resistance warrants further studies.

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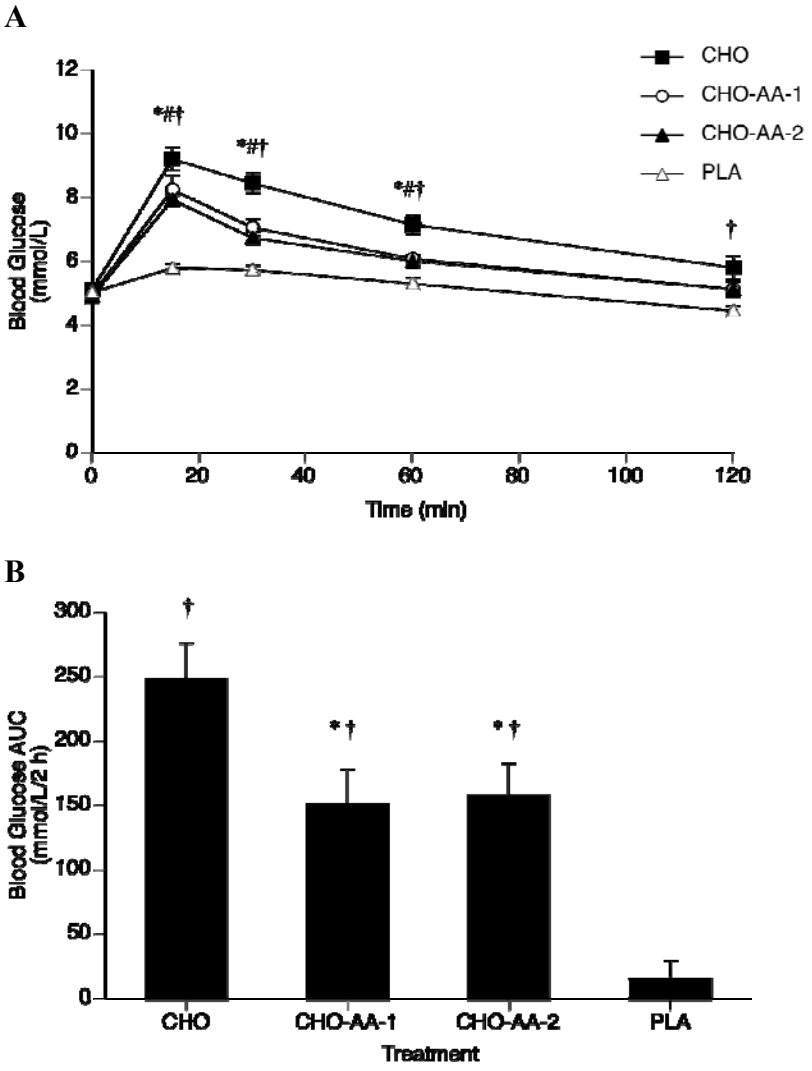

Figure 3.1. (A) Exp-1 blood glucose during the OGTT. Values are means ± SE. *, $P < 0.05$ CHO vs. CHO-AA-1; #, $P < 0.05$ CHO vs. CHO-AA-2; †, $P < 0.05$ PLA vs. all other treatments. (B) Exp-1 blood glucose AUC during the OGTT. Values are means ± SE. *, $P < 0.05$ vs CHO; †, $P < 0.05$ PLA vs. all other treatments.

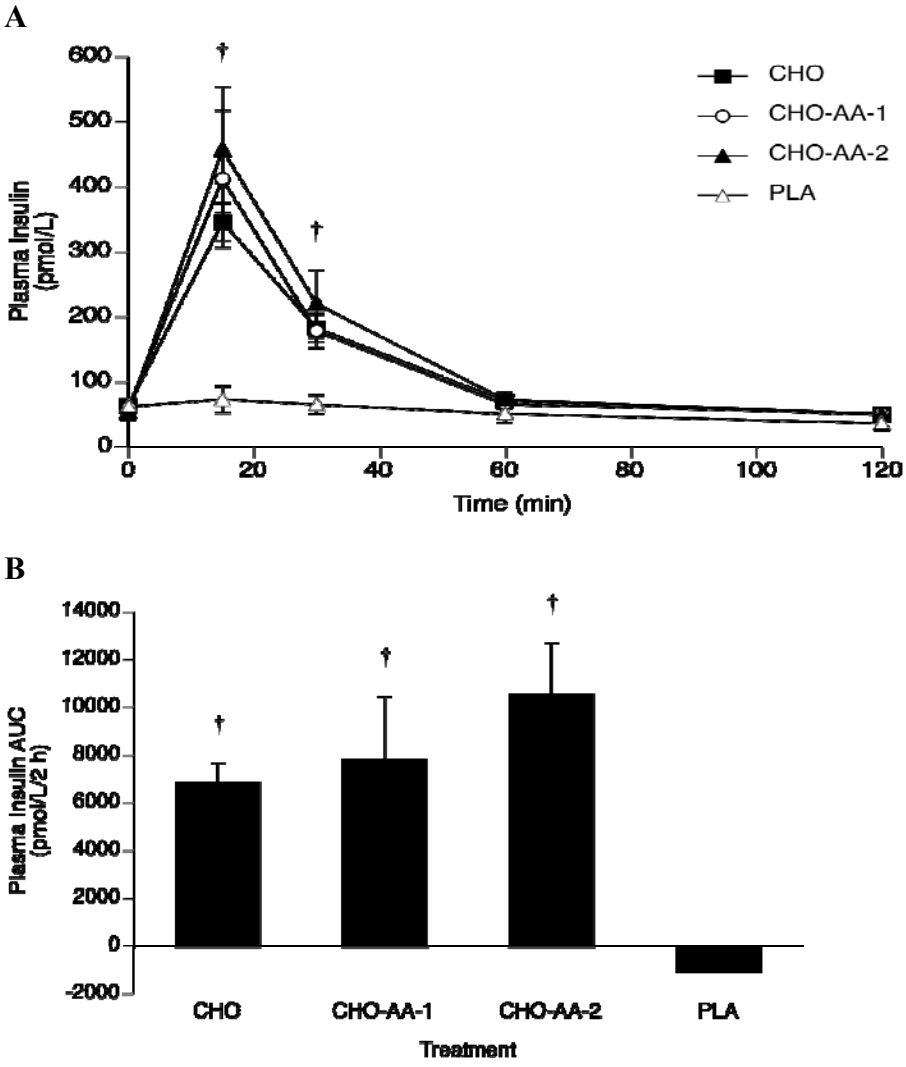

Figure 3.2. (A) Exp-1 plasma insulin during the OGTT. Values are means ± SE. †, $P < 0.05$ PLA vs. all other treatments. (B) Exp-1 plasma insulin AUC during the OGTT. Values are means ± SE. †, $P < 0.05$ PLA vs. all other treatments.

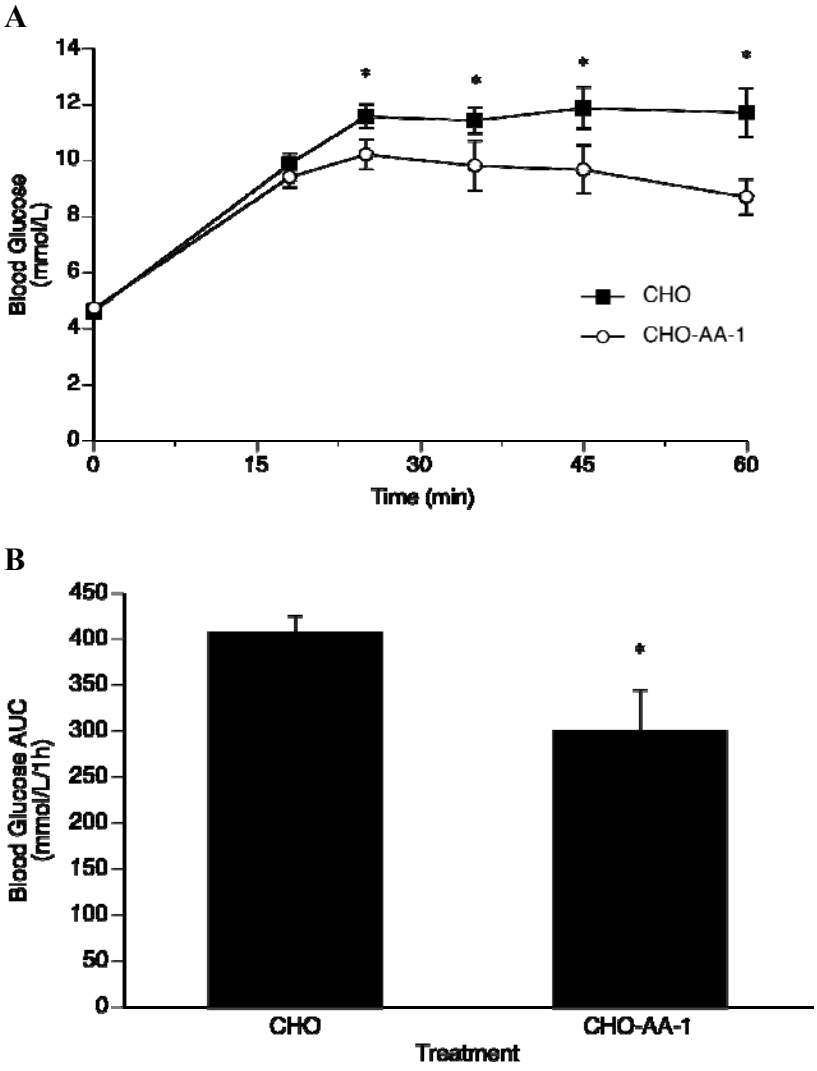

Figure 3.3. (A) Exp-2 blood glucose during the OGTT. Values are means ± SE. *, $P < 0.05$ CHO vs CHO-AA-1. (B) Exp-2 blood glucose AUC during the OGTT. Values are means ± SE. *, $P < 0.05$ vs CHO.

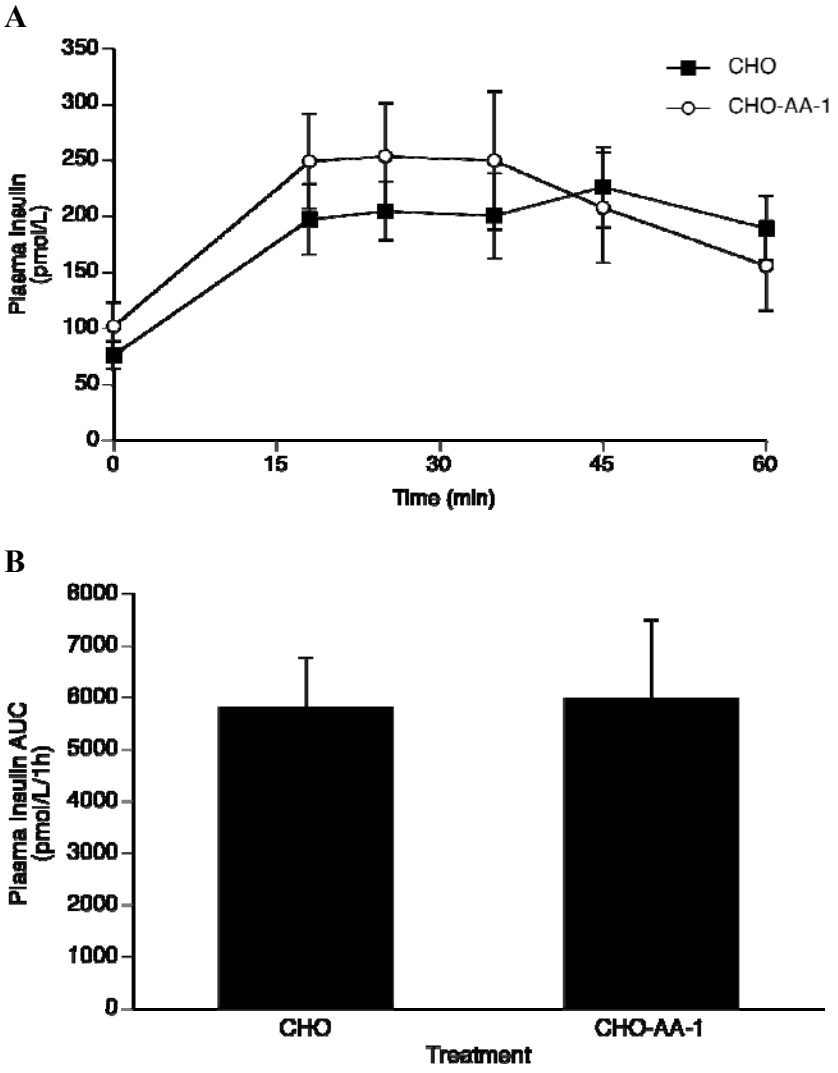

Figure 3.4. (A) Exp-2 plasma insulin during the OGTT. Values are means ± SE. (B) Exp-2 plasma insulin AUC during the OGTT. Values are means ± SE.

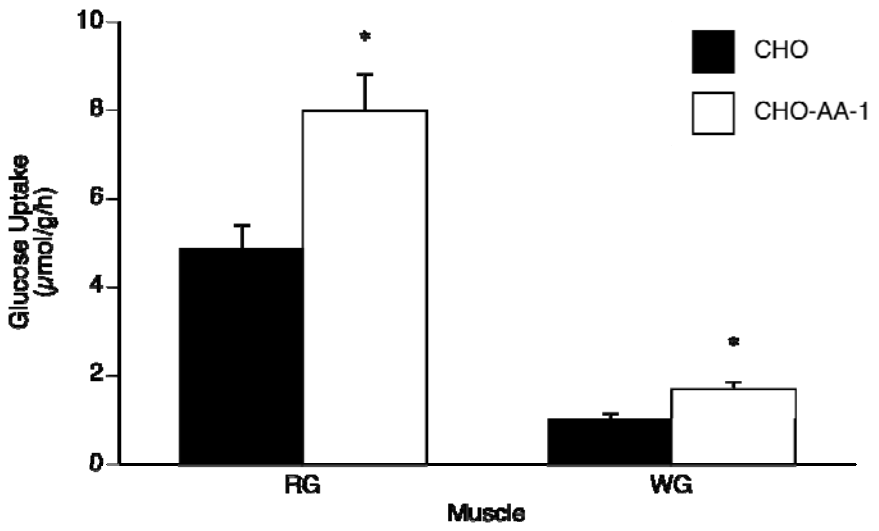

Figure 3.5. Exp-2 glucose uptake in the red and white gastrocnemius during the OGTT. Values are means ± SE. *, $P < 0.05$ CHO vs CHO-AA-1.

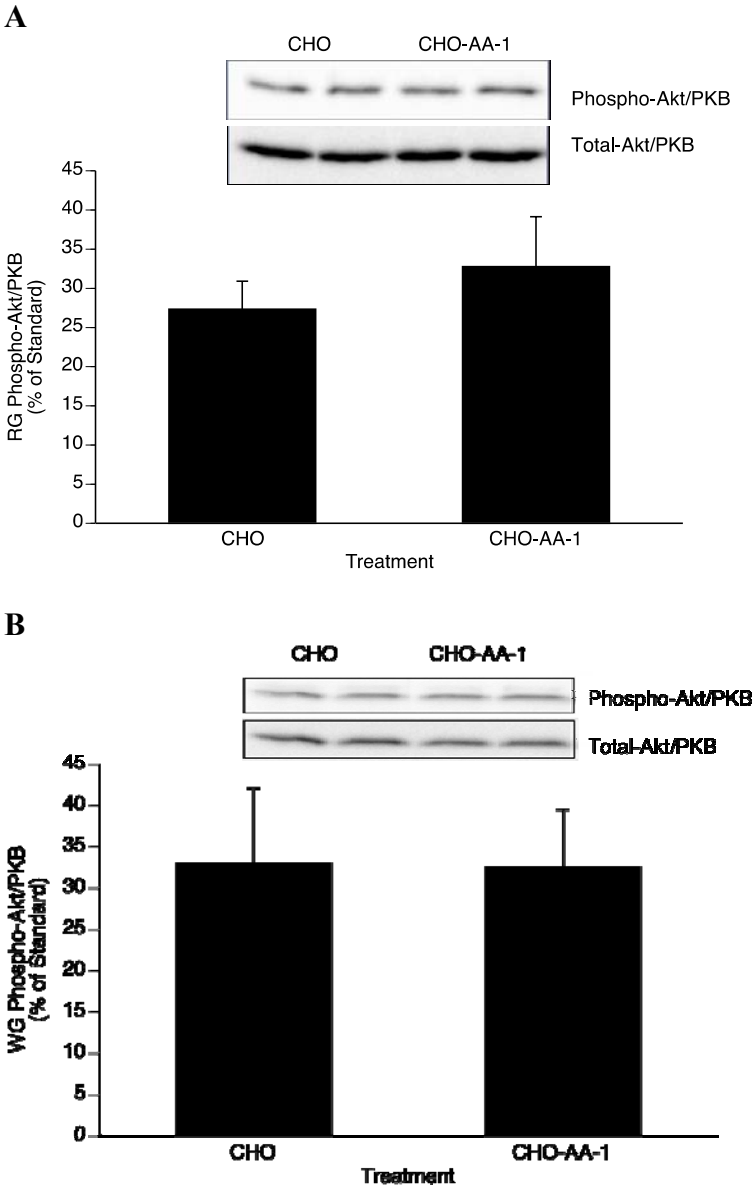

Figure 3.6. (A) Red gastrocnemius and (B) white gastrocnemius Akt/PKB Thr-308 phosphorylation and total protein concentration following the OGTT for Exp-2. Values are means ± SE.

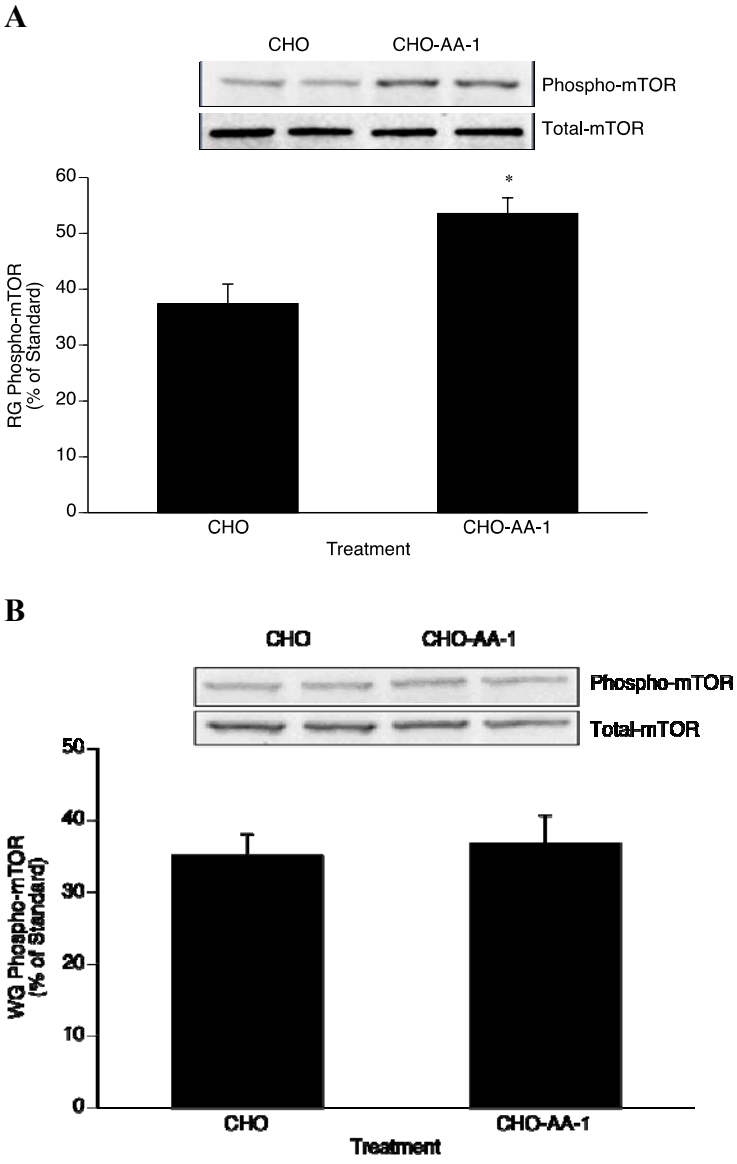

Figure 3.7. (A) Red gastrocnemius and (B) white gastrocnemius mTOR Ser-2448 phosphorylation and total protein concentration following the OGTT for Exp-2. Values are means $\pm$ SE. *, $P < 0.05$ vs CHO.

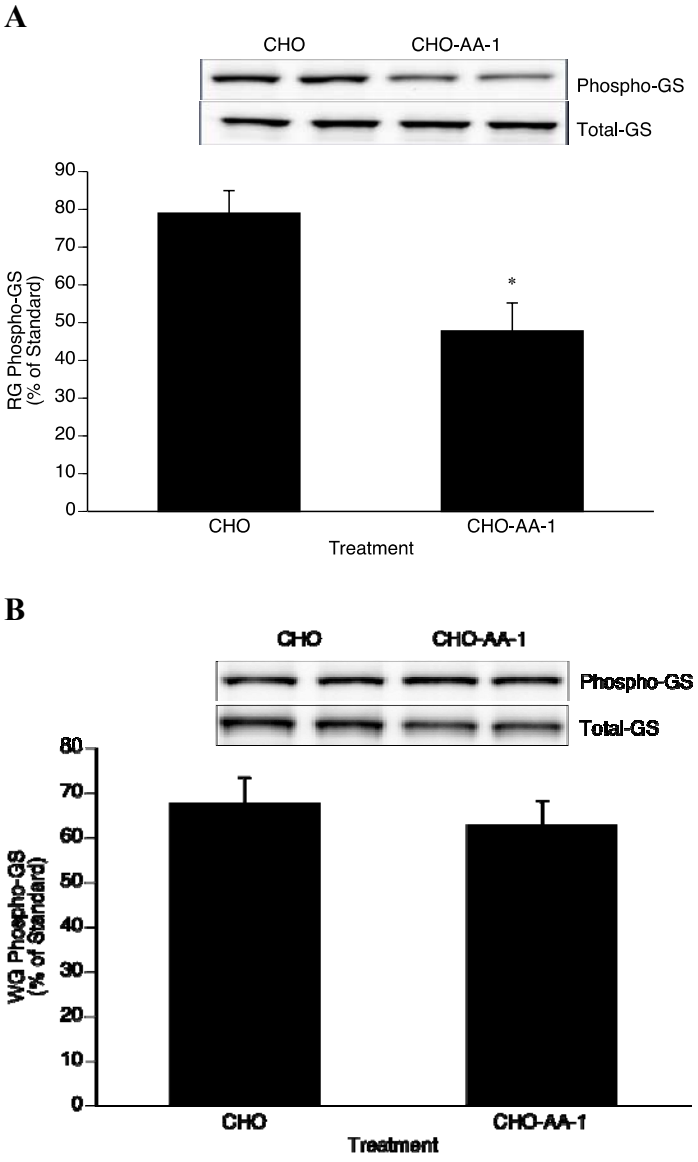

Figure 3.8. (A) Red gastrocnemius and (B) white gastrocnemius GS Ser-641 phosphorylation and total protein concentration following the OGTT for Exp-2. Values are means $\pm$ SE. *, $P < 0.05$ vs CHO.

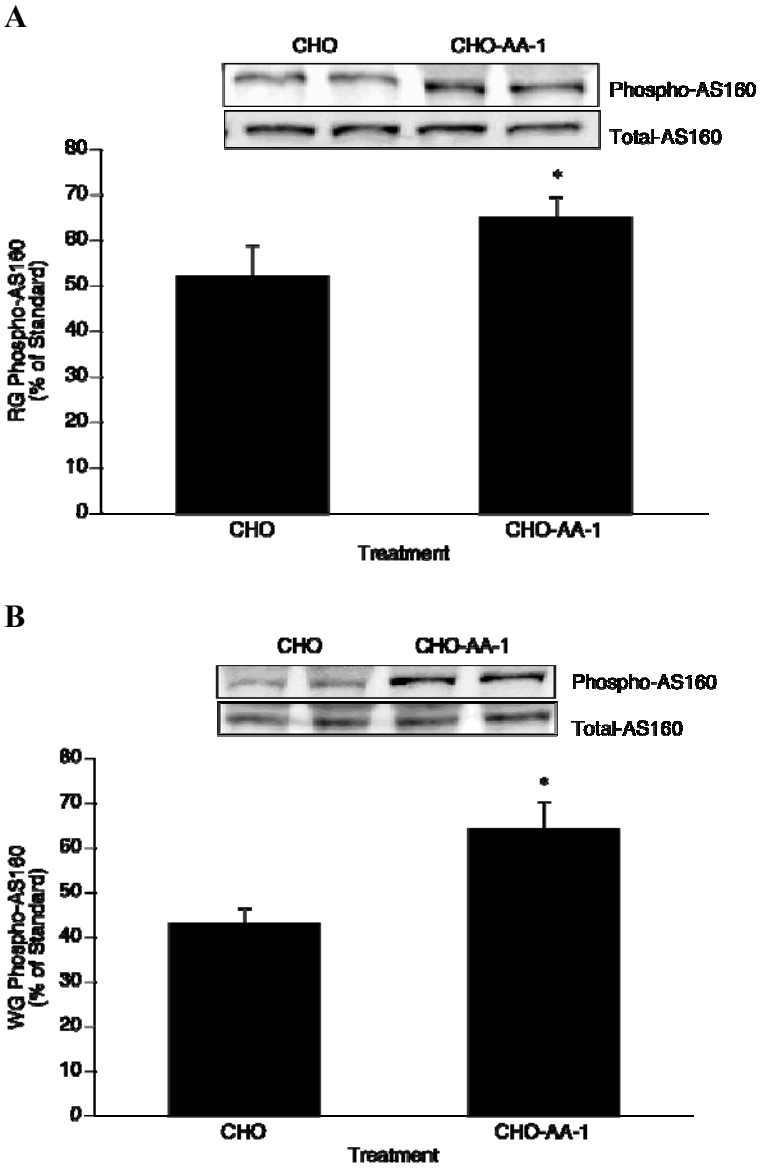

Figure 3.9. (A) Red gastrocnemius and (B) white gastrocnemius AS160 Thr-642 phosphorylation and total protein concentration following the OGTT for Exp-2. Values are means $\pm$ SE. *, $P < 0.05$ vs CHO.

Chapter IV: An amino acid mixture enhances insulin-stimulated glucose uptake and GLUT4 translocation in perfused rodent hindlimb muscle

ABSTRACT

The purpose of this study was to investigate whether an amino acid mixture increases glucose uptake across perfused rodent hindlimb muscle in the presence and absence of a submaximal insulin concentration. Sprague Dawley rats were separated into one of four treatment groups: amino acid mixture with submaximal insulin (AA-sINS), submaximal insulin (sINS), amino acid mixture with no insulin (AA) or basal (BAS). Glucose uptake was greater for both insulin-stimulated treatments compared to the non-insulin-stimulated treatment groups. In the presence of insulin, amino acids increased the rate of glucose uptake compared to a perfusate with no amino acids, but had no impact on uptake in the absence of insulin. Phosphatidylinositol 3-kinase (PI 3-kinase) activity was greater for both insulin-stimulated treatments compared to the non-insulin-stimulated treatment groups, but the amino acids had no additional impact on its activity. However, Akt substrate of 160 kDa (AS160) phosphorylation was increased by the amino acids in the presence of insulin, but not in the absence of insulin. Plasma membrane GLUT4 protein concentration was greater in the rats treated with insulin compared to no insulin in the perfusate. In the presence of insulin, amino acids enhanced GLUT4 translocation to the plasma membrane, but had no affect in the absence of insulin. Collectively, these findings suggest that the beneficial effects of an amino acid mixture on skeletal muscle glucose uptake, in the presence of a submaximal insulin concentration, are due to an increase in AS160 phosphorylation and plasma membrane associated GLUT4 and independent of PI 3-kinase activation.

INTRODUCTION

Insulin is the primary regulator of glucose homeostasis. The concentration of blood glucose at any given time is determined by the entry of glucose into the blood from the digestive system, its removal by glucose-sensitive tissues and its release from the liver. In the postprandial state, a rise in blood glucose stimulates the release of insulin from the pancreas. Insulin controls blood glucose within a narrow range by simultaneously increasing peripheral glucose uptake, primarily by the skeletal muscle, and decreasing liver glucose output. Recently, our laboratory (3, 12) as well as others (5, 7, 19) have reported that the glucose uptake process can be improved by amino acid supplementation.

Amino acids have recently been reported to increase skeletal muscle glucose uptake *in vivo* (3, 5, 7, 11) and *in vitro* (5, 12, 18, 19). Of the amino acids, the branched-chain amino acids isoleucine and leucine have received the most attention. Although these amino acids have been shown to significantly lower blood glucose, the exact mechanism remains elusive. Using the isolated muscle technique Nishitani et al. (19) demonstrated that leucine-induced glucose uptake was significantly decreased when the incubation medium included specific inhibitors for either phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream target atypical protein kinase C (aPKC) in the absence of insulin. Doi et al. (5) also reported that isoleucine-induced glucose uptake in C_2C_{12} myotubes was inhibited in the presence of a specific inhibitor of PI 3-kinase. Although it appears that PI 3-kinase is an important signal in amino acid-stimulated glucose uptake, it has also been reported that PI 3-kinase activity may be impaired by amino acids (2, 26). Therefore, in the presence of amino acids, the activity of PI 3-kinase, and how this kinase effects downstream proteins, requires further investigation.

In a recent report we showed that rats gavaged with an amino acid mixture had an improved glucose response to an oral glucose challenge compared to rats gavaged with carbohydrate only (3). In addition we demonstrated that the improved glucose tolerance was associated with enhanced Akt substrate of 160 kDa (AS160) phosphorylation and increased skeletal muscle glucose uptake (3). These findings raised the question as to the role PI 3-kinase and GLUT4 translocation, critical steps for blood glucose clearance, play in the increased glucose uptake. Furthermore, the *in vivo* study did not allow us to control the release of both carbohydrate and amino acids from the gut, nor could we precisely control the insulin levels in order to assess the independent and/or combined affect of insulin and the amino acid mixture. Therefore, in the present investigation, we used the hindlimb perfusion technique in order to control the amounts of carbohydrate, amino acids and insulin to which the muscle was exposed. This technique also enabled us to observe any alterations that could be attributed to the singular and combined effects of insulin and the amino acid mixture. Thus, the purpose of this investigation was to determine if an amino acid mixture increases glucose uptake in the perfused rodent hindlimb in the presence and absence of insulin, and if so, to determine if differences in glucose uptake were associated with differences in PI 3-kinase activity and GLUT4 translocation to the plasma membrane.

MATERIALS AND METHODS

Animal care and housing. Twenty-nine male Sprague Dawley rats approximately 7 weeks old were obtained from Harlan (Indianapolis, IN). Upon arrival rats were randomly assigned to one of four groups: amino acid with submaximal insulin (AA-sINS, n = 9), submaximal insulin (sINS, n = 7), amino acid with no insulin (AA, n = 7) or basal (BAS, n = 6). Rats were housed 2 per cage and provided standard laboratory

chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21° C, with an artificial 12:12 h light-dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conformed to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Hindlimb Perfusion. All animals were tested at approximately 9 weeks old. After an overnight fast rats were subjected to the hindlimb perfusion technique. Details for the surgical preparation and hindlimb perfusion technique have been described previously (10, 22). Perfusate flow was directed only to the right hindlimb by cannulating the right iliac artery and vein to the tip of the femoral artery of the rat. After the cannulas were in place the rat was euthanized by a cardiac injection (65 mg/kg body weight) of sodium pentobarbital. The cannulas were then placed in line with the perfusion system and the right hindlimb was washed out with Krebs-Henseleit buffer (pH 7.4) containing 2 mM pyruvate at a rate of 6 ml/min. Following the 10 min washout period the arterial line was switched to the perfusate containing 2 mM pyruvate, 6 mM glucose, 2 mM mannitol, 0.2 μCi/ml 2-[^{3}H] deoxyglucose (2-DG) and 0.15 μCi/ml [^{14}C(U)]-sucrose (^{14}C-sucrose) in Krebs-Henseleit buffer (pH 7.4) with the flow rate set at 4 ml/min. For the insulin-stimulated treatment groups (AA-sINS and sINS), 200 μU/ml insulin was added to both the washout buffer and perfusion medium. For the amino acid treatment groups (AA-sINS and AA) 2 mM of an amino acid mixture was also added to both the washout buffer and perfusate medium. The amino acid mixture was described previously (3) and contained 5.28 mg cysteine, 3.36 mg methionine, 6.68 mg valine, 944.8 mg isoleucine and 6.68 mg leucine per 50 ml solution. The perfusions were performed at 37° C and continued for a total of 25 minutes, at which time the right

gastrocnemius was excised, freeze clamped in liquid nitrogen, and stored at −80° C for later analysis.

Muscle glucose uptake. Rates of 2-DG uptake were determined in mixed gastrocnemius muscle samples. Muscle was weighed and dissolved in 1 N potassium hydroxide (KOH) by incubating for 15 min at 65° C. Samples were then vortexed and incubated for an additional 5 min. An equal volume of 1 N hydrochloric acid (HCl) was then added to neutralize the samples. Next, an aliquot of neutralized muscle sample was added to a vial containing 6 ml Bio-Safe II counting cocktail (Research Products International, Mount Prospect, IL). Arterial perfusate samples were taken during the hindlimb perfusion and treated the same as the muscle homogenate. Duplicate samples were counted in a liquid scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA) preset for simultaneous counting of [^{3}H] and [^{14}C] DPM. Quenching was determined by counting prepared standards. The accumulation of intramuscular 2-DG was indicative of muscle glucose uptake. Intramuscular 2-DG was calculated by subtracting the concentration of 2-DG in the extracellular space from the total muscle 2-DG concentration. The extracellular space for each muscle sample was determined by measuring the [U-^{14}C] mannitol concentration in the muscle homogenate.

PI 3-kinase activity. For IRS-1 associated PI 3-kinase activity, approximately 150 mg of muscle was homogenized (1:9) in an ice-cold homogenization buffer (pH 7.4) containing 20 mM HEPES, 2 mM EGTA, 50 mM sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerolphosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM Benzamidine, and 0.5 mM sodium orthovandate (Na_3VO_4) with a glass tissue grinder pestle (Corning Life Sciences, Acton, MA). The homogenate was then centrifuged at 14,000 x g for 10 min at 4° C. Aliquots of the supernatant were stored

at -80° C for later analysis. The protein concentration of the homogenate was determined using the Lowry method (16).

One milligram of sample protein was immunoprecipitated with 8 μg of anti-IRS-1 (Millipore, Billerica, MA) and homogenization buffer overnight at 4° C. Protein-A Sepharose (PRO-A) beads were then prepared by washing once with phosphate triton azide (PTA) and twice with homogenization buffer and centrifuged at 14,000 x g for 5 min at 4° C between each wash. After the final wash, the packed PRO-A beads were resuspended in homogenization buffer at a 1:1 dilution. One hundred microliters of PRO-A slurry was added to the anti-IRS-1 immunoprecipitates for 1.5 h at 4° C with rotation. Following the incubation, samples were centrifuged at 14,000 x g for 10 min at 4° C. The immunocomplex was then washed successively with the following: Buffer A (10% IGEPAL, 100 mM Na_3VO_4, 1 M DTT, phosphate buffered saline (PBS)), Buffer B (1 M Tris-HCl (pH 7.5), 2 M lithium chloride ($LiCl_2$), 100 mM Na_3VO_4, 1 M DTT), and Buffer C (1 M Tris-HCl (pH 7.5), 5 M sodium chloride (NaCl), 100 mM Na_3VO_4, 10 mM EDTA, 1 M DTT). Washing was performed once in buffers A and B and twice in buffer C. The packed beads were then diluted 1:1 in a buffer provided in the kit with 1 M DTT, 10 mM adenosine-5'-triphosphate (ATP) and 100 μM $PI(4,5)P_2$ substrate added to it. The substrate was incubated for 3 h and the product $PI(3,4,5)P_3$ was detected by an ELISA kit (catalogue# K1000s, Echelon Biosciences Inc., Salt Lake City, UT). The amount of phosphatidylinositol (3,4,5)-triphosate (PIP3) produced by extracted PI 3-kinase from the sample was proportional to the PI 3-kinase activity.

For the determination of total PI 3-kinase activity, approximately 150 mg of mixed gastrocnemius muscle was homogenized (1:9) in ice-cold 0.5 M TCA. The homogenate was then centrifuged at 1,500 rpm for 5 min at 4° C. The supernatant was discarded and the pellet was resuspended in 3 ml of 5% TCA/1 mM EDTA. The protein

concentration was then determined using the Lowry method (16). One milligram of protein was then subjected to the extraction protocol in accordance to the manufacturer's instructions. The extracted substrate was incubated for 1 h and the product PI(3,4,5)P_3 was detected using an ELISA kit (catalogue# K2500s, Echelon Biosciences Inc.). The amount of PIP3 produced by extracted PI 3-kinase from the sample was proportional to the total PI 3-kinase activity.

AS160 Phosphorylation. Approximately 150 mg of muscle was homogenized and the protein concentration of each sample determined as described above. Sample protein (60 μg) was combined with an equal amount (1:1) of Laemmli sample buffer (125 mM tris, 20% glycerol, 2% SDS, 0.008% bromophenol blue, pH 6.8) and boiled for 5 min (14). Next, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins separated on an 8% resolving gel for 1.5 h. The resolved proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer unit and blocked in 7% nonfat dry milk in Tris-Tween-buffered saline (NFDM/TTTBS) for 1 h at room temperature. The membranes were then incubated with affinity purified anti-phospho-AS160 (Thr-642) (Millipore, Billerica, MA) at 4° C. Following the overnight incubation the membranes were washed for 3, 5-min washes in TTBS then incubated for 2 h at room temperature with the species-specific secondary antibody. The membranes were then washed with 5, 8-min washes with TTBS and antibody binding was visualized by enhanced chemiluminescence in accordance to the manufacturer's instructions (Perkin Elmer, Boston, MA). Images were captured by using a charge-coupled device camera in a ChemiDoc system (BioRad, Hercules, CA) and saved to a computer. Density of the bands were quantified with Quantity One analysis software (BioRad) and expressed as a percentage of a standard run on each gel.

After the phosphorylation of AS160 was determined, the primary phosphorylated-antibody was stripped from the membrane to determine alpha-tubulin protein concentration in order to demonstrate equal protein loading across samples. Membranes were placed in a stripping solution containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris base (pH 6.7) and heated at 60° C for 1 h. Membranes were washed for 3, 15-min washes with TTBS to remove the stripping solution. The primary and secondary antibody incubation times, washing, exposure and quantification were the same as that described for AS160 phosphorylation.

Plasma membrane fractionation. The plasma membrane was isolated as described previously (15, 23). Briefly, a portion of the gastrocnemius was homogenized in an ice cold buffer (8 x wt/vol) containing 20 mM HEPES (pH 7.2), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na_3VO_4, 10% glycerol, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin A and 1 mM PMSF. The homogenate was centrifuged at 33,000 rpm for 30 min at 4° C and the supernatant was collected as the crude homogenate fraction. The pellet was resuspended in an ice-cold buffer (4 x wt/vol) in which 1% Trition X was added. The resuspended pellet was then centrifuged at 11,000 rpm for 10 min at 4° C. The supernatant, representing the plasma membrane fraction, was collected. In order to determine plasma membrane purity the enzymatic activity of the membrane marker 5'-nucleotidase in the plasma membrane fraction was compared to its activity in the crude homogenate as described previously (24). The method of Fiske and SubbaRow (9) was used for determination of 5'nucleotidase activity. The specific activities and purity indexes for the plasma membrane marker 5'-nucleotidase are displayed in Table 4.1. The protein concentration for the plasma membrane and crude homogenate was determined using the Lowry method (16).

Plasma membrane GLUT4 protein concentration. The plasma membrane GLUT4 protein concentration was determined from plasma membrane fractions obtained from mixed gastrocnemius muscle samples. Sample protein was subjected to SDS-PAGE and the protein was separated on a 12% resolving gel. Next, the resolved proteins were transferred to a PVDF membrane using a semidry transfer unit and blocked in NFDM-TTBS saline for 1 h at room temperature. Using a visible molecular weight marker (Bio-Rad) as a guide the PVDF membranes were cut into an upper and lower membrane section. The upper membrane section was probed with affinity purified anti-sodium-potassium-ATPase (Na^+K^+-ATPase) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4° C. The Na^+K^+-ATPase pump is a ubiquitous plasma membrane marker used to demonstrate equal protein loading across samples. The lower membrane section was probed with affinity purified anti-GLUT4 antibody (donated by Dr. Samuel Cushman, National Institute of Diabetes and Digestive Kidney Disease, Bethesda, MD) overnight at 4° C. The secondary antibody, incubation times, washing, exposure and quantification were similar to that described above.

Statistical analysis. A one-way analysis of variance (ANOVA) was performed on all data. When a significant F-ratio was obtained, a Fisher's Least Significant Difference post hoc test was performed to identify statistically significant differences ($P < 0.05$) between means. A sequential regression analysis was performed to determine the relationship between AS160, GLUT4 and muscle glucose uptake. Statistical analyses were completed using SPSS software (SPSS Inc., Chicago, IL) and all values expressed as means $\pm$ standard error (SE).

RESULTS

Animal characteristics. There were no significant differences in body mass among treatment groups (AA-sINS 293.7 $\pm$ 6.5 g; sINS 291.4 $\pm$ 3.1 g; AA 288.1 $\pm$ 5.7 g; BAS 284.8 $\pm$ 7.4 g) on the day of testing.

Muscle glucose uptake. Rates of 2-DG uptake in mixed gastrocnemius muscle samples were elevated for the AA-sINS and sINS treatments compared to the AA and BAS treatment groups (Figure 4.1). Furthermore, rates of glucose uptake were greater in the AA-sINS treatment group compared to sINS. However, under non-insulin-stimulated conditions, the addition of amino acids to the perfusate had no affect on glucose uptake compared to BAS.

PI 3-kinase activity. For both IRS-1 associated and total PI 3-kinase, there was greater activity for the insulin-stimulated treatments compared to the non-insulin-stimulated treatment groups. (Figure 4.2A-B). However, among the insulin-stimulated treatment groups, there was no difference observed between AA-sINS and sINS treatment groups. Similarly, among the non-insulin stimulated treatment groups, there was no difference between AA and BAS treatment groups.

Western blotting. Insulin-stimulation resulted in a significantly increased AS160 Thr-642 phosphorylation compared to the non-insulin-stimulated treatment groups (Figure 4.3). Moreover, AS160 phosphorylation was increased in the AA-sINS treatment compared to the sINS treatment group. There was no difference between AA compared to BAS treatment groups.

Insulin-stimulation resulted in a significant elevation in plasma membrane GLUT4 protein concentration compared to the non-insulin-stimulated treatment groups (Figure 4.4). The concentration of GLUT4 at the plasma membrane was also significantly greater in the AA-sINS treatment compared to the sINS treatment group.

There was, however, no difference in GLUT4 plasma membrane concentration in AA compared to BAS treatment groups.

Using sequential multiple regression analysis, we regressed AS160 and GLUT4 on skeletal muscle glucose uptake and found that the change in the coefficient of determination (ΔR^2) due to AS160 was 0.716 and 0.044 for GLUT4 (Figure 4.5). The total variance of glucose uptake explained by the model was 76%.

DISCUSSION

The primary objective of the present study was to test the ability of our amino acid mixture to increase skeletal muscle glucose uptake in the presence and absence of a submaximal insulin-stimulating concentration using the hinblimb perfusion technique. To achieve this objective, we compared the effects of perfusing the hindlimb musculature with four different experimental treatments, AA-sINS, sINS, AA or basal BAS on glucose uptake. Our study results demonstrated that skeletal muscle glucose uptake during the hindlimb perfusion was significantly greater for the AA-sINS treatment compared to sINS. This finding confirms our previous studies reporting that our amino acid mixture enhances glucose uptake both *in vitro* (12) and *in vivo* (3) in the presence of insulin. We were able expand this in the present investigation by demonstrating that the increase in amino acid-stimulated glucose uptake was directly related to an increase in AS160 phosphorylation and plasma membrane associated GLUT4 protein concentration. We, however, could not confirm our previous data reporting that our amino acid mixture increases skeletal muscle glucose uptake in the absence of insulin (12).

Activation of the insulin receptor results in cellular mechanisms that directly mediate the number of glucose transporters translocated to the plasma membrane. Once insulin is bound to its receptor, the insulin receptor phosphorylates tyrosine residues on

IRS-1. In turn, phosphorylated IRS-1 binds and activates PI 3-kinase which then catalyzes the conversion of phosphatidylinositol (4,5)-bisiphosate (PIP2) to PIP3. This is a critical step in classical insulin signaling as blocking PI 3-kinase is known to inhibit insulin-stimulated glucose uptake. The subsequent activation of the downstream proteins protein kinase B (Akt/PKB), aPKC and AS160 leads to GLUT4 translocation to the plasma membrane. Thus, glucose uptake is a highly coordinated process and is highly correlated with the number of GLUT4 transporters located at the plasma membrane (8, 13, 17).

In the present investigation we found that insulin had a strong stimulatory effect on skeletal muscle glucose uptake. Submaximal insulin-stimulation resulted in approximately a 3 fold difference in glucose uptake compared to the non-insulin-stimulated conditions. Furthermore, the combination of amino acids and insulin resulted in a 36% increase in glucose uptake compared to insulin alone. Doi et al, (5) and Nishitani et al. (19) also demonstrated a beneficiary impact of amino acids on glucose uptake. Doi et al. (5) reported that glucose uptake was enhanced when isoleucine was added to C_2C_{12} myotubes. But when LY294002, a potent PI 3-kinase inhibitor, was added to the same medium, a significant decrease in glucose uptake was observed. Furthermore, Nishitani et al. (19) reported that leucine stimulated glucose uptake across the isolated soleus muscle preparation but leucine-induced glucose uptake was inhibited in the presence of LY294002. These *in vitro* studies provide support for the involvement of PI 3-kinase in amino acid-stimulated skeletal muscle uptake. These *in vitro* studies also infer that amino acids induce glucose uptake via PI 3-kinase leading to GLUT4 translocation. To the best of our knowledge, there are no previous studies investigating the link between amino acids, PI 3-kinase activity, GLUT4 translocation and skeletal

muscle glucose uptake. Therefore, in an effort to determine how amino acids increase skeletal muscle glucose uptake we first measured PI 3-kinase activity.

In the present study, both IRS-1 associated and total PI 3-kinase activity were significantly increased in the presence of insulin compared to a perfusate with no insulin. In fact, there was approximately a 3 fold difference between insulin and non-insulin stimulated PI 3-kinase activity, which correlates well with the approximately 3 fold difference found with glucose uptake. However, adding the amino acid mixture to the perfusate had no further impact on PI 3-kinase activity in the presence and absence of insulin, suggesting that amino acid-stimulated glucose uptake is independent of PI 3-kinase. A major difference between our current study and the *in vitro* studies discussed above (5, 19) is that we actually measured PI 3-kinase activity, rather than determine the necessity of PI 3-kinase in amino acid-stimulated glucose uptake. Although amino acids do not appear to enhance PI 3-kinase activity, it is possible that there is a permissive amount of PI 3-kinase activity required for this process.

The exact role PI 3-kinase plays in amino acid-stimulated glucose uptake has been elusive. *In vitro* studies using inhibitors to block PI 3-kinase suggest that the protein kinase may be involved in this process (5, 19). But it must be noted that the inhibitor used in these studies, LY294002, blocks all classes of PI 3-kinase (27). Nevertheless, it is difficult to ascertain its importance when it has been demonstrated that amino acids attenuate the length of time PI 3-kinase is activated (2, 21, 26), suggesting that over time glucose uptake would be reduced as well, possibly via an mTOR mediated negative feedback mechanism on IRS-1 (26). Baum et al. (2) reported that rats gavaged with either carbohydrate or a carbohydrate plus leucine supplement had similar IRS-1 associated PI 3-kinase activity 15 min post supplementation. However, from 30-90 min post supplementation, carbohydrate treated rats displayed significantly higher kinase

activity compared to carbohydrate plus leucine. Interestingly, glucose uptake was still normal despite the decreased amino acid-induced PI 3-kinase activity suggesting that the early activation of the kinase may have been sufficient to initiate glucose uptake (2). In the present investigation, there was no difference in IRS-1 associated or total PI 3-kinase activity between sINS and AA-sINS treatment groups perfused for a total of 35 min. Because the activity of PI 3-kinase was not enhanced by amino acids, these results suggest that PI 3-kinase may only play a permissive role in amino acid-stimulated glucose uptake. In agreement, it has been reported that leucine-stimulated PI 3-kinase activity is independent of its association with IRS-1 in L6 muscle cells (21). Thus, future studies investigating alternate proteins linking amino acids to PI 3-kinase and/or different classes of PI 3-kinase are warranted.

In our effort to determine a mechanism for amino acid-induced skeletal muscle glucose uptake we next looked at the impact of our amino acid mixture on AS160 phosphorylation. AS160, a distal signal in the insulin signaling cascade, prevents GLUT4 translocation. Once phosphorylated, however, its inhibitory effect on GLUT4 translocation is removed and GLUT4 is able to move from an intracellular storage pool to the plasma membrane. We previously reported that our amino acid mixture increased AS160 phosphorylation *in vivo* (3) and *in vitro* (12) and the increase in AS160 phosphorylation was associated with an amino acid-induced increase in muscle glucose uptake. In agreement, the current investigation found that perfusing our amino acid mixture also increased AS160 phosphorylation. But this affect was only observed in the presence of a submaximal insulin concentration, not in the absence of insulin. Using the isolated muscle preparation we previously demonstrated that amino acids increase both AS160 phosphorylation and muscle glucose uptake without Akt/PKB activation (12). This finding would agree with our current PI 3-kinase data as Akt/PKB is activated by PI

3-kinase. Therefore, this suggests that amino acids increase the phosphorylation of AS160 via a mechanism independent of the insulin signaling pathway.

Using carbon tetrachloride (CCl_4) induced liver cirrhotic rats, administration of leucine and isoleucine lowered the blood glucose response to an oral glucose challenge, increased skeletal muscle glucose uptake and increased plasma membrane associated GLUT4 protein concentration compared to control (20). The positive impact amino acids had on glucose homeostasis occurred without differences in insulin secretion. However, amino acid administration could have resulted in *in vivo* changes that increased the effectiveness of insulin and not directly increase GLUT4 translocation. In the present investigation, plasma membrane GLUT4 protein concentration was significantly increased when insulin was added to the perfusate. Furthermore, the number of glucose transporters located at the plasma membrane was even greater when the perfusate contained both insulin and our amino acid mixture demonstrating amino acids have a direct role in regulating GLUT4 translocation. Enhanced amino acid-stimulated GLUT4 translocation fits well with our earlier work as we have previously reported our amino acid mixture increased AS160 phosphorylation and glucose uptake (3, 12) and adds to the data because we are now able to demonstrate a direct link between AS160, GLUT4 and glucose uptake. Using sequential regression analysis we found that the increase in GLUT4 translocation could account for approximately 4% of the increase in glucose uptake. While the increase in AS160 could account for approximately 72% of the increase in glucose uptake. This indicates that the amino acid induced increase in AS160 phosphorylation is responsible for GLUT4 translocation.

Although we found a strong correlation between AS160, GLUT4 translocation and muscle glucose uptake, given what is currently know about insulin signaling, it is interesting that the increase in plasma membrane GLUT4 occurred without an increase in

IRS-1 associated PI 3-kinase activity. There are several possibilities that may explain this observation. First of all, the energy sensor 5'-AMP-activated protein kinase (AMPK) can also recruit GLUT4 to the plasma membrane. However, this appears unlikely as it has been demonstrated that isoleucine-stimulated glucose uptake is not mediated through an AMPK dependent mechanism (6). Secondly, the mammalian target of rapamycin (mTOR) which is activated by amino acids and plays a role in amino acid-stimulated protein synthesis (1) is another possibility. However, this also appears unlikely as several studies have shown that blocking mTOR with its specific inhibitor, rapamycin, has no impact on amino acid-stimulated glucose uptake (5, 19). Thirdly, a downstream signal from PI 3-kinase, PKC may play a role in the amino acid induced increase in plasma membrane GLUT4 protein concentration as it has been reported that the activation of PKC may regulate GLUT4 translocation through AS160 (25). *In vitro* studies report that blocking PKC with its specific inhibitor, GF109203X, results in significantly reduced amino acid-stimulated glucose uptake (5, 19). Therefore, it is possible that amino acids activate PKC, in a yet to be determined manner, to enhance GLUT4 translocation.

Despite the fact perfusing rodent hindlimb muscle with our amino acid mixture and insulin significantly increased glucose uptake, we did not observe increased amino acid-stimulated glucose uptake in the absence of insulin. In contrast, using the isolated muscle preparation, our laboratory previously demonstrated that our amino acid mixture increased glucose uptake across the epitrochlearis muscle in the absence of insulin (12). This is in agreement with earlier *in vitro* studies reporting that leucine (19), isoleucine (5) and isoleucine-leucine dipeptides (18) increased glucose uptake independent of insulin. Furthermore, in *in vivo* studies, insulin appears to only play a permissive role in amino-acid stimulated glucose uptake. Oral administration of isoleucine significantly lowered blood glucose and increased skeletal muscle glucose uptake but did not raise plasma

insulin levels compared to rats treated with saline (6, 7). Consistent with these reports, our laboratory found that there was no difference in insulin secretion between rats orally gavaged with an amino acid mixture and those gavaged with a carbohydrate supplement, but the amino acid mixture resulted in a reduced blood glucose response (3). Collectively, these findings suggest that the effects of amino acids on glucose uptake are independent of insulin. In addition, when we added a submaximal and a maximal insulin concentration to the isolated muscle incubation medium, we found that the insulin and amino acid effects were additive (12). This would suggest that not only are the effects of amino acids on glucose uptake independent of insulin, but also suggests that amino acids may act independently from the classical insulin signaling cascade.

The discrepancy between studies that report a stimulatory effect of amino acids on glucose uptake in the absence of insulin, and the present study in which we observed no effect, is likely due to the experimental model employed in the respective studies. For example, the fidelity of the hindlimb perfusion technique may not be as good so small differences between treatments may be more difficulty to identify. Those reporting that amino acids enhanced glucose uptake in the absence of insulin were *in vitro* studies and performed with cell culture and the isolated muscle preparation. Both the isolated muscle and hindlimb perfusion techniques are widely accepted approaches for metabolic studies, and the advantage and disadvantages of each have been extensively review elsewhere (4). Nevertheless, blood flow is not a factor for the *in vitro* isolated muscle preparation. The relatively small and thin soleus (19) and epitroclearis (12) muscles allow excellent muscle fiber access to amino acids in solution. In contrast, the perfusion technique uses an intact circulatory system and exposes the entire hindlimb musculature to the different experimental treatments. Insulin is a vasodilator and therefore may influence perfusate flow through the capillary beds during hindlimb perfusion. Thus, we may not have

observed increased amino acid-stimulated muscle glucose uptake in the absence of insulin because blood flow was shunted to the large blood vessels of the hindlimb and not all the muscle capillary beds were open. In contrast, when a submaximal insulin concentration was added to the perfusate, blood flow to the muscle capillary beds was improved in a nitric oxide dependent manner, contributing to the significantly greater uptake observed in the presence of insulin.

In summary, our amino acid mixture was found to increase skeletal muscle glucose uptake compared to a perfusate with no amino acids in the presence of insulin. However, the present study would suggest that insulin is required for the improvement in glucose tolerance as a perfusate containing the amino acid mixture, but no insulin, was found not to be anymore beneficial compared to BAS. While the mechanism by which amino acids work is not fully understood, our findings demonstrated that amino acid-stimulated skeletal muscle glucose uptake was not due to increased PI 3-kinase activity. Rather, the amino acid induced improvements in glucose tolerance appear due to increased AS160 phosphorylation and enhanced GLUT4 translocation to the plasma membrane by a process that may be unique to amino acid stimulation.

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	AA-sINS	sINS	AA	BAS
PM	24.0 ± 1.7 *	21.1 ± 1.5 *	24.0 ± 2.1 *	21.7 ± 0.7 *
CR	3.5 ± 0.3	3.0 ± 0.2	3.4 ± 0.4	3.1 ± 0.1
Purity index	7.0 ± 0.4	7.1 ± 0.5	7.4 ± 0.6	7.1 ± 0.3

Table 4.1. 5'-nucleotidase activity in the plasma membrane and crude homogenate. 5'-nucleotidase activity is expressed as μmol/mg protein/min. Values are means are ± SE. PM, plasma membrane; CR, crude homogenate. * $P < 0.05$ significanlty different from CR.

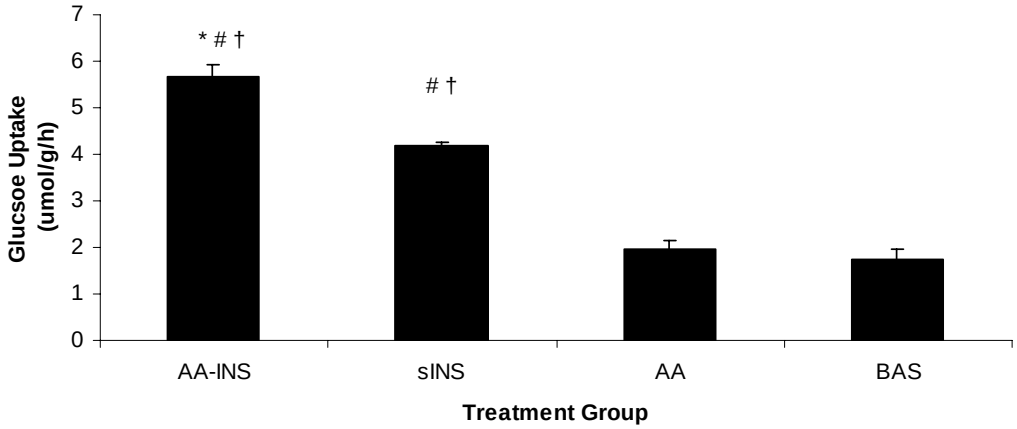
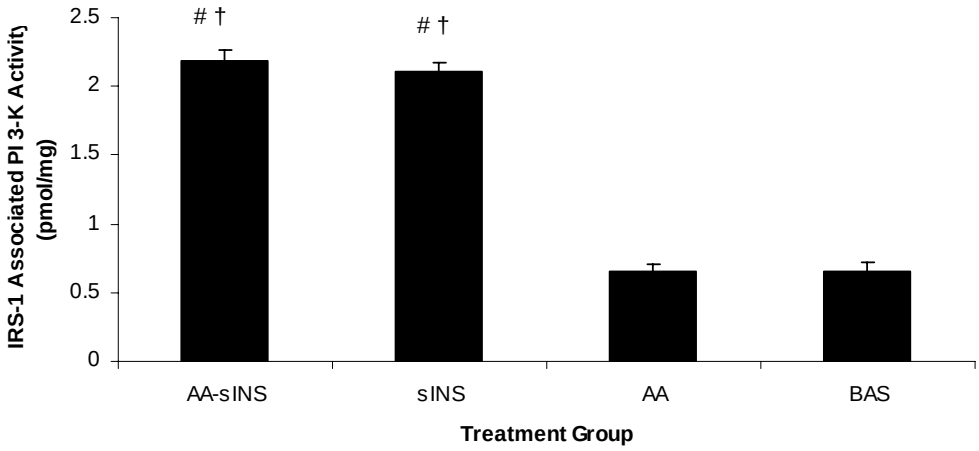


Figure 4.1. Glucose uptake in mixed gastrocnemius samples obtained from rats subjected to the hindlimb perfusion technique. Values are means $\pm$ SE. *, $P < 0.05$ vs sINS, $^{\#}$, $P < 0.05$ vs AA †, $P < 0.05$ BAS.

A



B

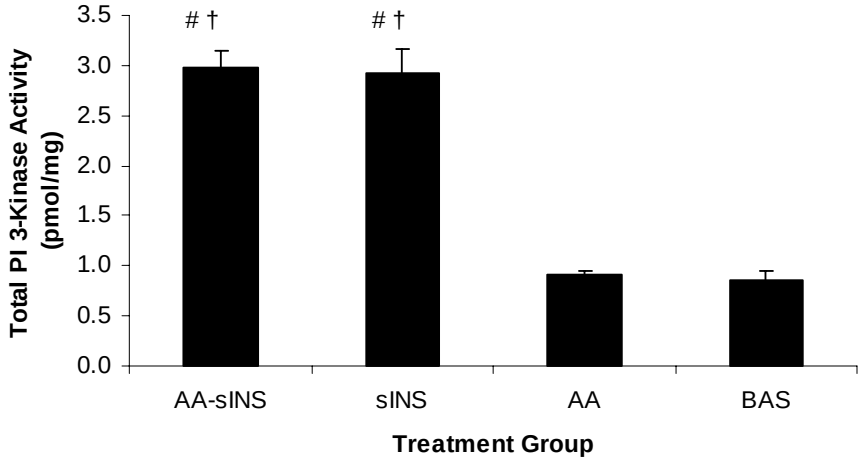


Figure 4.2. (A) IRS-1 associated PI 3-kinase activity and (B) total PI 3-kinase activity in mixed gastrocnemius samples obtained from rats subjected to the hindlimb perfusion technique. Values are means $\pm$ SE. $^{\#}$, $P < 0.05$ vs AA †, $P < 0.05$ BAS.

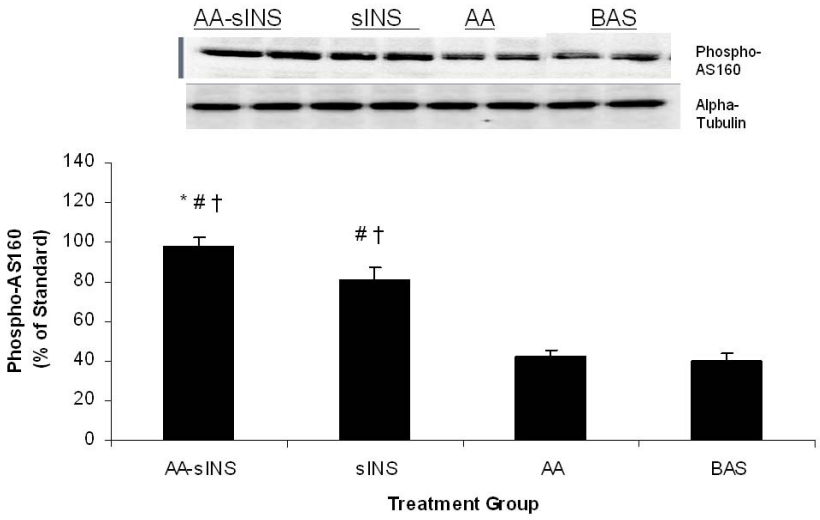


Figure 4.3. AS160 Thr-642 phosphorylation in mixed gastrocnemius samples obtained from rats subjected to the hindlimb perfusion technique. Values are means ± SE. *, $P < 0.05$ vs sINS, $^{\#}$, $P < 0.05$ vs AA †, $P < 0.05$ BAS.

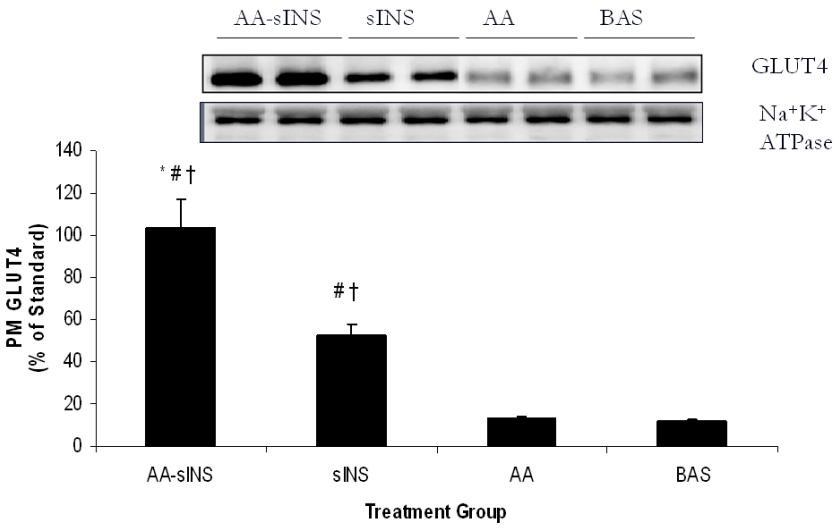


Figure 4.4. Plasma membrane GLUT4 protein concentration in mixed gastrocnemius samples obtained from rats subjected to the hindlimb perfusion technique. AA-sINS, perfusate contained 6 mM carbohydrate, 200 μU/ml insulin and a 2 mM amino acid mixture; sINS, perfusate contained 6 mM carbohydrate and 200 μU/ml insulin; AA, perfusate contained 6 mM carbohydrate and 2 mM amino acid mixture; BAS, perfusate contained 6 mM carbohydrate. Values are means $\pm$ SE. *, $P < 0.05$ vs sINS, $^{\#}$, $P < 0.05$ vs AA †, $P < 0.05$ BAS.

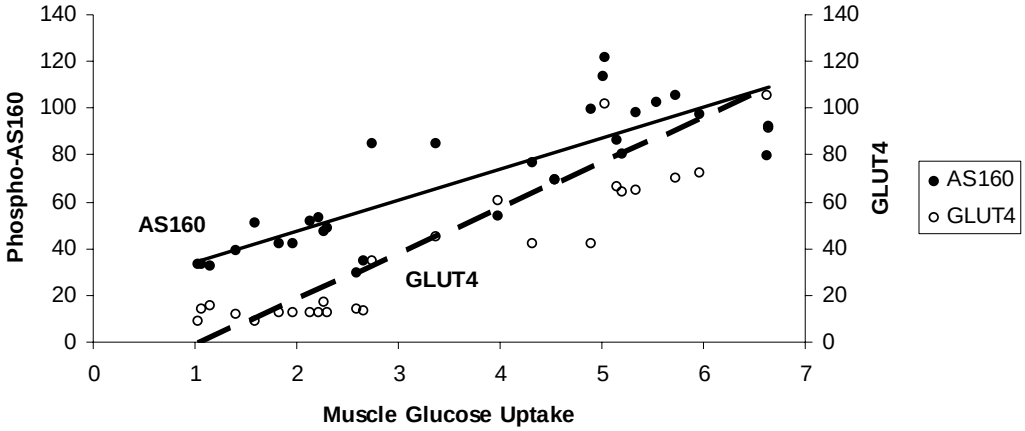


Figure 4.5. Relationship between the increase in muscle glucose uptake associated with phosphorylated AS160 (R^2 = 0.72) and plasma membrane GLUT4 (R^2 = 0.65) protein concentration.

Chapter V: An amino acid mixture improves glucose tolerance and lowers insulin resistance in the obese Zucker rat

ABSTRACT

The purpose of this investigation was to test an amino acid mixture on glucose tolerance in obese Zucker rats (experiment [Exp]-1) and determine whether differences in blood glucose were associated with alterations in muscle glucose uptake (experiment [Exp]-2). Exp-1 rats were gavaged with either carbohydrate (OB-CHO), carbohydrate plus an amino acid mixture (OB-AA-1), carbohydrate plus an amino acid mixture with increased leucine concentration (OB-AA-2) or water (OB-PLA). The glucose response in OB-AA-1 and OB-AA-2 were similar, and both were lower compared to OB-CHO. There was no difference in insulin among treatment groups. Rats in Exp-2 were gavaged with either carbohydrate (OB-CHO), carbohydrate plus an amino acid mixture (OB-AA-1) or water (OB-PLA). Lean Zucker rats were gavaged with carbohydrate (LN-CHO). Fifteen minutes after gavage a radiolabeled glucose analogue was infused through a catheter previously implanted in the right jugular vein. Blood glucose was significantly lower in OB-AA-1 compared to OB-CHO while the insulin responses were similar. Glucose uptake was greater in OB-AA-1 compared with OB-CHO, and similar to that in LN-CHO in red gastrocnemius muscle (5.15 $\pm$ 0.29, 3.8 $\pm$ 0.27, 5.18 $\pm$ 0.34 umol/100 g /min, respectively). Western blot analysis showed that Akt substrate of 160 kDa (AS160) phosphorylation was enhanced for OB-AA-1 and LN-CHO compared to OB-CHO. These findings suggest that an amino acid mixture improves glucose tolerance in an insulin resistant model and that these improvements are associated with an increase in skeletal muscle glucose uptake possibly due to improved intracellular signaling.

INTRODUCTION

Type 2 diabetes is a disease that is characterized by severe insulin resistance of insulin sensitive tissues such as skeletal muscle, liver and adipose tissue. When the body is presented with a glucose challenge, insulin is released into the general circulation and binds to its specific receptor. The binding of insulin to its plasma membrane receptor triggers a series of phosphorylative events which leads to the removal of glucose from the circulation via glucose transporter 4 (GLUT4). Because skeletal muscle is the primary site for postprandial glucose disoposal (10, 20), it is therefore, important in regulating glucose homeostasis.

Dietary supplementation has become popular in many populations for improving, for example, athletic performance and one's general well being. It is often advertised as a natural alternative to a pharmacologic based approach. Thus, dietary supplementation is particularly attractive for diabetics and those with insulin resistance as supplementation can be used for weight and/or glycemic control. Recent investigations have focused on amino acid supplementation as a means of controlling the postprandial glucose response. Of the amino acids, the branched-chain amino acids (BCAAs) have generated much research interest as these have been shown to markedly lower blood glucose (11, 12, 27). These recent reports demonstrated both leucine and isoleucine to be the most potent of the BCAAs in enhancing skeletal muscle glucose uptake. Thus, the ability of amino acid supplementation to lower blood glucose holds great clinical significance for individuals with impaired glucose tolerance such as that observed in insulin resistant and diabetic states.

Obese Zucker rats are a widely accepted research model for the study of insulin resistance. These rats are characterized by a genetic disorder that causes them to be obese and have impaired insulin resistance. Obese Zucker rats display severe

hyperinsulinemia, glucose intolerance and elevated plasma insulin response to a glucose challenge, similar to that observed in insulin resistant human subjects. Our lab has recently shown that an amino acid mixture improves glucose tolerance, both *in vivo* and *in vitro*, in healthy, non-insulin resistant tissue (5, 22) and this enhancement appears to be due, at least in part, to changes in insulin cellular signaling and increased skeletal muscle glucose uptake. However, little is known as to the effect of amino acid supplementation in an insulin resistant model. Therefore, the purpose of this investigation was to determine if an amino acid mixture improves glucose tolerance in Obese Zucker rats (experiment [Exp]-1), and if so, are alterations in glucose tolerance due to differences in skeletal muscle glucose uptake and cell signaling (experiment [Exp]-2).

MATERIALS AND METHODS

Animals. Exp-1: Thirty-five female obese Zucker rats approximately 7 weeks old were obtained from Charles River (Wilmington, MA). The obese Zucker rats were randomly assigned to one of four groups: obese-carbohydrate (OB-CHO, n = 9), obese-carbohydrate plus a 5 amino acid mixture (OB-AA-1, n = 9), obese-carbohydrate plus a 5 amino acid mixture with increased leucine concentration (OB-AA-2, n = 9) or obese-placebo (OB-PLA, n = 8). Rats were housed individually and provided standard laboratory chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21° C, and an artificial 12:12 h light-dark cycle set. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conformed to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Exp-2: Eighteen female obese Zucker rats and six lean Zucker rats approximately 7 weeks old were obtained from Charles River. The obese Zucker rats were then randomly assigned to either obese-carbohydrate (OB-CHO, n = 6), obese-carbohydrate plus a 5 amino acid mixture (OB-AA-1, n = 7) or obese-placebo (OB-PLA, n = 5). Lean Zucker rats were assigned to the carbohydrate group (LN-CHO, n = 6). Rats were housed individually and provided standard laboratory chow and water ad libitum as described for Exp-1. Animal care and university approval was similar to that described for Exp-1.

Experimental protocol. Exp-1: Following 2 weeks of acclimation, rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior the testing to become accustomed to the experimental procedures. After a 12 h fast each rat was wrapped in a towel and the tip of their tail cut and bled prior the oral glucose tolerance test (OGTT). Rats were orally gavaged (8 ml/kg body weight) with 1 of 4 solutions: 1) OB-CHO (22.5% glucose), OB-AA-1 (amino acid mixture in 22.5% glucose), OB-AA-2 (amino acid mixture with increased leucine concentration in 22.5% glucose), or OB-PLA (distilled water). The amino acid mixture contained 5.28 mg cysteine, 3.36 mg methionine, 6.68 mg valine, 944.8 mg isoleucine and 6.68 mg leucine per 50 ml solution. The leucine concentration was increased to 50 mg/50 ml for the OB-CHO-AA-2 treatment. Blood was taken from the tail immediately before the gavage and 15, 30, 60 and 120 min after. Immediately after rats were released from the towel wrap and returned to their cage until the next blood collection. Blood was collected in one test tube containing ethylenediaminetetraacetic acid (EDTA) (24 mg/ml, pH 7.4) and another with 10% perchloric acid (PCA) for later analysis. Immediately after the 120 min blood sample, rats were sacrificed with a cardiac injection of sodium pentobarbital (65 mg/kg body weight).

Exp-2: Following 2 weeks of acclimatization, rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior testing to become accustomed to the experimental procedures. Four days before the OGTT rats were prepared for surgical implantation of a jugular vein catheter. Rats were placed under isoflurane gas anesthesia in order to perform the jugular vein catheterization. Briefly, the right jugular vein was accessed above the clavicle by making a small incision over the ventral thorax. After removing the surrounding tissue to expose the jugular vein, ligatures were placed around the vein and a catheter containing heparinized saline inserted into the vein and guided down to the right atrium. The catheter was then guided from the thorax and out a small incision on the head. To ensure that the catheter was in the proper position a small amount of blood was drawn, flushed and heparinized saline stored in the catheter to prevent clotting. The incision sites over the thorax and head were then sutured and the catheter was sealed with a silicone patch to prevent backflow of the stored heparinized saline. A small strip of Velcro with a hole was sutured over the incision behind the head. The catheter was then threaded through the Velcro and the remaining catheter was coiled and placed between 2 Velcro strips for protection (2).

Each morning leading up to the experimental day the rats had their catheter flushed to maintain catheter patency and gavaged with saline to familiarize them with the OGTT procedure. Four days post surgery, and following a 12 h fast rats were wrapped in a towel and the tip of their tail cut and bled as discussed in the above section. The rats were then orally gavaged (8 ml/kg body weight) with either 22.5% glucose (OB-CHO and LN-PLA), an amino acid mixture in 22.5% glucose (OB-AA-1) or distilled water (OB-PLA). The amino acid mixture was described previously in Exp-1. Fifteen min after the gavage, a bolus containing 40 μCi/kg body weight [^{3}H] 2-deoxyglucose (2-DG) and 20 μCi/kg body weight [U-^{14}C] mannitol was injected by syringe via the jugular

catheter. The tail was bled and blood collected as describe above at 2, 10, 20 and 45 min after the radioactive tracers were infused for later analysis. Immediately after the 45 min blood sample, corresponding to 60 min after gavage, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight) at which time the red and white gastrocnemius were excised, freeze clamped in liquid nitrogen and stored at -80° C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital.

Blood analysis. A drop of blood was used to measure blood glucose with a portable glucose analyzer (One Touch Ultra 2; LifeScan Inc., Milpitas, CA). EDTA samples were then centrifuged 14,000 x g for 10 min at 4° C and the plasma insulin determined by a radioimmunoassay kit according to the manufactures instructions (Linco, St. Charles, MO).

Muscle glucose uptake. Rates of 2-DG uptake were determined in both red and white gastrocnemius muscle samples. Approximately 80-100 mg of muscle were dissolved in 1 N potassium hydroxide (KOH) by incubating for 15 min at 65° C, vortexed then incubated for an additional 5 min at 65° C. Next, an equal volume of 1 N hydrochloric acid (HCl) was added to the digested samples and vortexed to neutralize the samples. To determine the specific activity of the blood, an aliquot of the PCA extract was added to 1 N KOH, then neutralized with an equal volume of 1 N HCL. A 300 µl aliquot of neutralized muscle and blood samples were then added to a vial containing 6 ml Bio-Safe II counting cocktail (Research Products International, Mount Prospect, IL). Duplicate samples were counted in a liquid scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA) preset for simultaneous counting of [^{3}H] and [^{14}C] DPM. Quenching was determined by counting prepared standards. The accumulation of intracellular 2-DG was indicative of muscle glucose uptake. The specific activity of the

blood for [^{3}H] and [^{14}C] was determined using the integral of the plasma 2-DG and [U-^{14}C] mannitol over the 15-60 min per glucose molecule. The extracellular space was calculated using the total muscle ^{14}C DPM and its specific activity. The intramuscular accumulation of 2-DG was calculated by subtracting its extracellular space DPM from its total muscle DPM divided by its specific activity.

Tissue processing. Approximately 60 mg of muscle was homogenized (1:9) in an ice-cold buffer (pH 7.4) containing 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 2 mM ethylene glycol tetraacetic acid (EGTA), 50 mM sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerolphosphate, 1 mM DL-Dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM Benzamidine, and 0.5 mM sodium Vanadate (1 ml/100 mg muscle) with a glass tissue grinder pestle (Corning Life Sciences, Acton, MA). The homogenate was then centrifuged at 14,000 x g for 10 min at 4° C. Thereafter aliquots of the supernatant were stored at -80° C for later analysis. The protein concentration of the homogenate was determined using the Lowry method (26).

Western blotting. The phosphorylation of protein kinase B (Akt/PKB), mTOR, Akt substrate of 160 kDa (AS160) and glycogen synthase (GS) were used as an indirect measurement of activity. Sample protein (100 μg for Akt/PKB, 70 μg mTOR and AS160 and 60 μg for GS) was combined with an equal amount (1:1) of Laemmli sample buffer (125mM tris, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.008% bromophenol blue, pH 6.8) (23) and boiled for 5 min. Next, sample proteins were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins separated on either an 8% (mTOR and AS160) or 12% polyacrylamide (Akt/PKB and GS) resolving gel for either 1 h (Akt/PKB) or 1.5 h (mTOR, AS160 and GS). The resolved proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane

using a semidry transfer unit and blocked in 7% nonfat dry milk in Tris-Tween-buffered saline (NFDM/TTTBS) for 1 h at room temperature. The membranes were then incubated with either affinity purified anti-phospho-Akt/PKB (Thr-308) (Cell Signaling Technology, Danvers, MA), anti-phospho-mTOR (Ser-2448) (Cell Signaling Technology), anti-phospho-AS160 (Thr-642) (Millipore, Billerica, MA) or anti-phospho-GS (Ser-641) (Cell Signaling Technology) overnight at 4° C. These phosphorylation sites were chosen as an indirect measurement of activity because they represent the primary sites of phosphorylation of the respective protein under insulin-stimulated conditions (15, 32, 33, 35). The primary antibodies were diluted to either 1:500 (phospho-Akt/PKB), 1:800 (phospho-AS160) or 1:1000 (phospho-mTOR and phospho-GS) in TTBS containing 2% NFDM. Following the overnight incubation the membranes were washed for 3, 5-min washes in TTBS then re-blocked in 7% NFDM for an additional 15 min. Next, membranes were washed in TTBS and then incubated for either 1 h (Akt/PKB and GS) or 2 h (mTOR and AS160) at room temperature with the species-specific (anti-rabbit) immunoglobulin G (IgG) secondary antibodies (Cell Signaling Technology). The secondary antibodies were diluted to either 1:750 (Akt/PKB and AS160), 1:900 (mTOR) or 1;2000 (GS) in TTBS containing 2% NFDM. The membranes were washed for 5, 8-min washes with TTBS and antibody binding was visualized by enhanced chemiluminescence in accordance to the manufacture's instructions (Perkin Elmer, Boston, MA). Images were captured by using a charge-coupled device camera in a ChemiDoc system (BioRad, Hercules, CA) and saved to a computer. Density of the bands quantified with Quantity One analysis software (BioRad) and expressed as a percentage of a standard run on each gel.

After the phosphorylation status of each protein had been determined, the primary phospho-antibody was stripped from the membrane to determine the total protein

concentration. Membranes were placed in a stripping solution containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris base (pH 6.7) and heated at 60° C for 1 h. Membranes were washed for 3, 15-min washes with TTBS to remove the stripping solution. The primary and secondary antibody concentrations, incubation times, washing and quantification were the same as that described for the determination of phosphorylation status.

Statistics. A two-way analysis of variance (ANOVA) was performed on the blood data (treatment x time). A one-way ANOVA was performed for the muscle tissue analysis. When a significant F-ratio was obtained, a Fisher's Least Significant Difference (LSD) post hoc test was performed to identify statistically significant differences ($P < 0.05$) between means. All statistical analyses were completed using SPSS software (SPSS Inc., Chicago, IL) and all values expressed as means $\pm$ standard error (SE).

RESULTS

Animal characteristics. For Exp-1, there were no significant differences in body mass among treatment groups (OB-CHO 336.6 $\pm$ 9.3; OB-AA-1 344.9 $\pm$ 6.5; OB-AA-2 338.2 $\pm$ 8.7; OB-PLA 333.5 $\pm$ 10.0) on the day of testing. For Exp-2, there was no difference in body mass between the obese Zucker rat treatment groups (OB-CHO 338.7 $\pm$ 5 g; OB-AA-1 340.3 $\pm$ 13.2 g; OB-PLA 329 $\pm$ 15.9 g) on the day of testing. However, the lean Zucker rats were significantly smaller (185 $\pm$ 6.9 g) than all obese Zucker treatment groups.

OGTT glucose and insulin. For both experiments, glucose and insulin concentrations were determined following a 12 h fast and during the OGTT. For Exp-1, there were no significant differences in fasting glucose or insulin levels among treatment groups. There was little change in the blood glucose response for OB-PLA throughout

the OGTT during Exp-1. This would indicate no undue stress on the animals throughout the OGTT. In Exp-1, blood glucose was significantly lower for OB-AA-1 and OB-AA-2 compared to OB-CHO at 15, 30, 60 and 120 min post supplementation (Figure 5.1A). The glucose area under the curve (AUC) was also lower for both the low and high amino acid treatments compared to carbohydrate alone in the obese Zucker rats (Figure 5.1B). Although there were no statistically significant differences in plasma insulin at any time point between OB-CHO, OB-AA-1 or OB-AA-2 (Figure 5.2A), both amino acid treatment groups had a greater insulin AUC compared to the carbohydrate only treatment (Figure 5.2B).

For Exp-2, there was no difference in fasting blood glucose levels among the obese treatment groups (Figure 5.3A). However, the fasting blood glucose of the lean rats was significantly lower compared to each obese treatment group. The blood glucose response for OB-PLA was crucial to this study as this measurement indicated that the rats were properly familiarized and had recovered from the surgical procedure. The small increase in blood glucose measured for OB-PLA was similar to the changes observed in the initial studies, suggesting that the rats had recovered from surgery and were not stressed by the OGTT procedure. The blood glucose response for LN-CHO was significantly lower compared to OB-CHO and OB-AA-1 at all time points post supplementation. Blood glucose was also significantly reduced for OB-AA-1 compared to OB-CHO at 27, 37, 47 and 60 min. The glucose AUC displayed a similar trend (figure 5.3B). OB-PLA was lower than all other treatment groups, and the glucose AUC was reduced in LN-CHO compared to the obese Zucker rat treatment groups. In addition, the glucose AUC was significantly lower in OB-AA-1 compared to OB-CHO.

There was no difference in fasting plasma insulin between the obese rat treatments in Exp-2 (Figure 5.4A). However, fasting insulin was significantly lower in

the lean rats compared to obese rats. The insulin for LN-CHO was lower than all obese treatments at each time point during the OGTT. OB-PLA, which was gavaged with distilled water, had little change in insulin over the 60 min OGTT, and their insulin was significantly lower than OB-CHO and OB-AA-1 at all time points. There was no difference in plasma insulin detected between OB-CHO and OB-AA-1 at 0, 17, 27, 47 or 60 min. When the insulin AUC was calculated there was no difference between OB-CHO and OB-AA-1 (figure 5.4B). The insulin AUC was significantly lower for LN-CHO compared to OB-CHO and OB-AA-1, but not different from OB-PLA.

Muscle glucose uptake. Rates of skeletal muscle 2-DG uptake for Exp-2 are shown in Figure 5.5A-B. Glucose uptake was significantly increased in all treatment groups compared to OB-PLA in the red gastrocnemius. Glucose uptake for OB-AA-1 and LN-CHO treatment groups were significantly increased compared to OB-CHO in the red gastrocnemius. Similar to that observed in the red muscle, glucose uptake was increased for all treatment groups compared to OB-PLA in the white gastrocnemius. However, there were no differences detected for rates of glucose uptake between OB-CHO, OB-AA-1 and LN-CHO treatment groups in the white gastrocnemius.

Protein concentration and phosphorylaiton status of signaling proteins. There was no difference between treatment groups in the total protein concentration for Akt/PKB, mTOR, GS or AS160 detected following acute supplementation in either the red or white gastrocnemius in Exp-2. The phosphorylation status for these proteins were then assessed as an indirect measurement of activity. There was also no difference between treatment groups for the phosphorylation of Akt/PKB (Figure 5.6A-B), mTOR (Figure 5.7A-B), or GS (Figure 5.8A-B) in both red and white gastrocnemius. However, treatment difference did exist in the red gastrocnemius for AS160 phosphorylation (Figure 5.9A). AS160 phosphorylation was significantly increased in OB-AA-1

compared to OB-CHO, and was similar to that in LN-CHO. While all treatment groups were significantly elevated above OB-PLA. In the white gastrocnemius, there was no difference in AS160 phosphorylation between, OB-CHO, OB-AA-1 and LN-CHO, but each was increased compared to OB-PLA (Figure 5.9B)

DISCUSSION

The primary finding of the present investigation was that our amino acid mixture consisting of isoleucine, leucine, cysteine, methionine and valine improved glucose tolerance in insulin resistant obese Zucker rats on an acute basis. This affect does not appear to be due to an elevated plasma insulin concentration. Rather, the improved glucose tolerance appears due to increased skeletal muscle glucose uptake and enhanced AS160 phosphorylation. Previously, our laboratory reported that the addition of an amino acid mixture significantly lowered the blood glucose response to an oral glucose challenge in Sprague Dawley rats. Using the radioactive glucose analogue 2-DG we then determined that the attenuated glucose response was due to enhanced skeletal muscle glucose clearance with no differences in the plasma insulin response between amino acid and carbohydrate treatment groups (5). Thus, the present investigation adds to what we previously reported in healthy, non-insulin resistant tissue, and demonstrates that the enhanced amino acid induced glucose uptake can be applied to an insulin resistant model as well.

Insulin is the primary regulator of glucose uptake during the postabsorptive state. In general, the greater the insulin response, the greater insulin stimulated glucose uptake. Therefore, ways to increase the insulin response have been investigated as a mean to increase the amount of glucose cleared by the muscle. Ingesting carbohydrates elicit rapid insulin secretion from the pancreas, however, co-ingesting carbohydrate with

proteins and/or amino acids has been shown to further elevate the increase insulin response (18, 29, 38, 39, 41). The branched-chain amino acid leucine in particular is known to stimulate *in vivo* insulin secretion (16, 18). With this in mind, for Exp-1, we increased the leucine concentration of the amino acid mixture from 0.13 mg/ml to 1 mg/ml. Despite increasing the leucine concentration of the amino acid mixture we did not observe a further increase in plasma insulin, nor did we observe an additional affect of the added leucine on blood glucose levels. Therefore, we proceeded to use the amino acid mixture containing the low leucine concentration for our second experiment. Possible reasons we were unable to elicit a greater insulin response by adding more leucine to the amino acid mixture could be due to the relatively low amount of leucine in the mixture, the rate amino acids were released into the gut or possibly because the insulin levels were already in excess in the obese Zucker rat. These findings are similar to what we reported previously in healthy non-insulin resistant tissue (5) and suggest that the lower blood glucose response to the carbohydrate plus amino acid mixture was not due to increased insulin secretion for the amino acid treatment groups.

Although we are unable to completely rule out the involvement of insulin for amino acid-stimulated glucose uptake using our *in vivo* model, it does appear that insulin does not play a significant role in this process. It is noteworthy that the primary ingredient of our amino acid mixture is the BCAA isoleucine, which has been shown to increase glucose uptake *in vivo* without a synergistic increase in insulin secretion in rats (11) and in human subjects (30). Lowered blood glucose response with amino acids, then, if not due to increased insulin concentrations, may be associated with improved intracellular signaling. Using the specific inhibitors for phosphatidylinositol 3-kinase (PI 3-kinase) and mTOR, Doi et al. (11) reported that isoleucine increased glucose uptake in C_2C_{12} myotubes via a PI 3-kinase dependent and mTOR independent mechanism. In

agreement, using an isolated soleus preparation, Nishitani et al. (27) found that leucnine-stimulated glucose uptake occurred via a PI 3-kinase and protein kinase C (PKC) dependent mechanism, and mTOR was not involved in this process. Collectively, these studies would suggest that amino acids improve intracellular signaling through the PI 3-kinase pathway, resulting in increased GLUT4 translocation to the plasma membrane and increased glucose removed by skeletal muscle. Thus, it has been demonstrated, in rats with liver cirrhosis, that both leucine and isoleucine increase skeletal muscle glucose uptake by increasing GLUT4 translocation to the plasma membrane (28).

Our previous investigation using Sprague Dawley rats found that the amino acid mixture stimulated muscle glucose uptake by enhancing intracellular signaling, specifically at GS and AS160 (5). It is noteworthy that our amino acid mixture decreased the glucose response to a carbohydrate load in a model in which blood glucose clearance was already optimal. Therefore, we wanted to investigate whether the amino acid mixture had a similar affect in an insulin resistant model, and if so, would the mechanisms for improvement be the same.

We first sought to determine the effect of insulin resistance on glucose uptake and the associated insulin signaling proteins. We found that the glucose uptake in the obese treatment group was reduced by 36% across the red gastrocnemius compared to the lean Zucker rats when treated with the carbohydrate supplement. This is in agreement with previous reports, and the insulin resistance of obese Zucker rats is believed due to a combination of insulin receptor and post receptor defects (6, 7, 8, 9, 34). In the present investigation the amino acid mixture resulted in a 35% increase in glucose uptake in obese Zucker rats compared to obese Zuckers treated with carbohydrate only. Interestingly, we observed a similar increase in skeletal muscle glucose uptake for both the LN-CHO and OB-AA, suggesting that the amino acid mixture was able to restore

glucose disposal in the insulin resistant rats, although it required a much greater insulin response. We did not, however, observed differences in the total protein expression between lean and obese Zucker rats for Akt, mTOR, GS or AS160. Thus, the reduced glucose uptake in the obese Zucker rats does not appear due to reduced total protein expression of these specific insulin signaling proteins (4, 19).

We also did not observe differences in the phosphorylaiton status between lean and obese Zucker rats for Akt, mTOR, or GS when treated with carbohydrate or the amino acid mixture. We did, however, find that the amino acid mixture significantly increased red gastrocnemius AS160 phosphorylation compared to OB-CHO. The phosphorylation and subsequent inhibition of AS160 by Akt/PKB is a critical step in GLUT4 translocation to the plasma membrane. Under basal conditions AS160 maintains a Rab protein in its inactive GDP-bound state. Upon insulin stimulation, AS160 is phosphorylated, allowing the conversion of the Rab protein to its activated GTP-bound form, enabling GLUT4 translocation and tissue glucose uptake (40). Thus, not being able to attribute the increase in AS160 phosphorylation to increased Akt/PKB phosphorylation is noteworthy, but in agreement with our findings in Sprague Dawley rats (5, 22). In our previous study testing the amino acid mixture in Sprague Dawley rats we reported a 61% increase in AS160 phosphorylation for rats gavaged with the amino acid mixture compared to carbohydrate alone (5). Nevertheless, the 37% increase in amino acid-stimulated AS160 phosphorylation translated to a 35% increase in glucose uptake in the present investigation, and the 61% increase in AS160 phosphorylation in Sprague Dawley rats resulted in a 59% increase in glucose uptake. The reasons why there was less of an affect of the amino acid mixture in obese Zuckers relative to Sprague Dawley rats is not clear, but it is likely related to the muscular and/or systemic effects of insulin resistance.

It is noteworthy that we did not observe a difference in glucose uptake between lean and obese Zucker rats treated with carbohydrate in the white gastrocnemius, suggesting that the white muscle was not insulin resistance. However, the glucose uptake in the white muscle was very low in both the obese and lean Zuckers relative to that observed in red muscle. The average glucose uptake in the white gastrocnemius for OB-CHO, OB-AA-1 and LN-CHO was 0.387 μmol/100 g/min compared to 4.72 μmol/100 g/min, respectively, in the red gastrocnemius. Therefore, rates of glucose uptake in white muscle were approximately $1/12^{th}$ of that seen in red muscle. Some possible reasons for this is that white muscle is more glycolytic, has fewer capillaries surrounding the muscle fiber (30), and has significantly less GLUT4 (3, 17, 22) compared to the more oxidative red muscle. As a result of these fiber type differences, it would be expected that there be reduced blood flow, insulin-stimulation and glucose uptake in the white gastrocnemius. Thus, it is also possible that the very low glucose uptake in the white muscle prevented us from detecting differences in uptake between OB-CHO, OB-AA-1 and LN-CHO.

Despite the positive results obtained in the present investigation using the amino acid mixture, some studies suggest that amino acid supplementation actually contributes to insulin resistance. It has been reported that circulating amino acids are elevated in obese individuals (13, 14) and that diets high in protein are associated with impaired glucose metabolism (24, 25). Thus, several studies propose that amino acids attenuate glucose uptake via a negative feedback mechanism through mTOR (36, 37). Accordingly, elevated amino acids activate the mTOR/p70 pathway which then inhibit PI 3-kinase through serine phosphorylation of insulin receptor substrate-1 (IRS-1) leading to skeletal muscle insulin resistance (36). Amino acids, especially the BCAA leucine, is a strong activator of mTOR signaling. However, isoleucine is not believed to activate mTOR (1). Since the predominate ingredient of our amino acid mixture is isoleucine,

rather than leucine, this may help explain the discrepancy among studies. Although leucine has been shown to increase glucose uptake it is feasible that relatively low concentrations of leucine are beneficial for glucose metabolism while high concentrations of leucine may impair this process (27). Nevertheless, future studies exploring the dose and temporal response relationship of leucine and glucose uptake are warranted.

In conclusion we demonstrated that an amino acid mixture enhanced skeletal muscle glucose uptake in obese Zucker rats during an oral glucose challenge. The effects of the amino acid mixture appear to be independent of insulin and due, at least in part, to increased AS160 phosphorylation. These finding suggest that the acute ingestion of an amino acid mixture consisting predominately of isoleucine can improve insulin resistance. However, further research is needed to determine the long term affects of amino acid supplementation on insulin resistance and whether the positive results found in the present investigation translates to humans and those with more severe insulin resistance such as that observed in type 2 diabetes.

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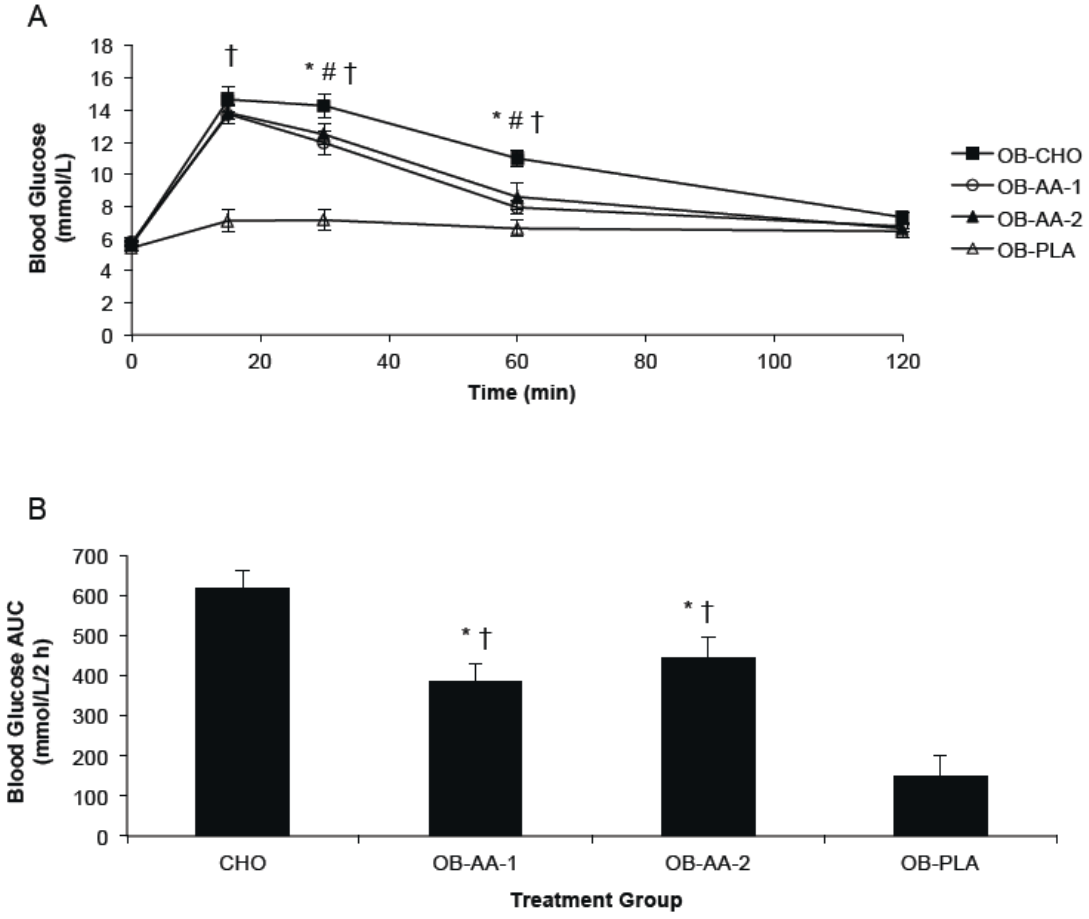

Figure 5.1. (A) Exp-1 blood glucose during the OGTT. Values are means ± SE. *, $P < 0.05$ OB-CHO vs. OB-AA-1; #, $P < 0.05$ OB-CHO vs. OB-AA-2; †, $P < 0.05$ OB-PLA vs. all other treatments. (B) Exp-1 blood glucose AUC during the OGTT. Values are means ± SE. *, $P < 0.05$ vs. OB-CHO; †, $P < 0.05$ OB-PLA.

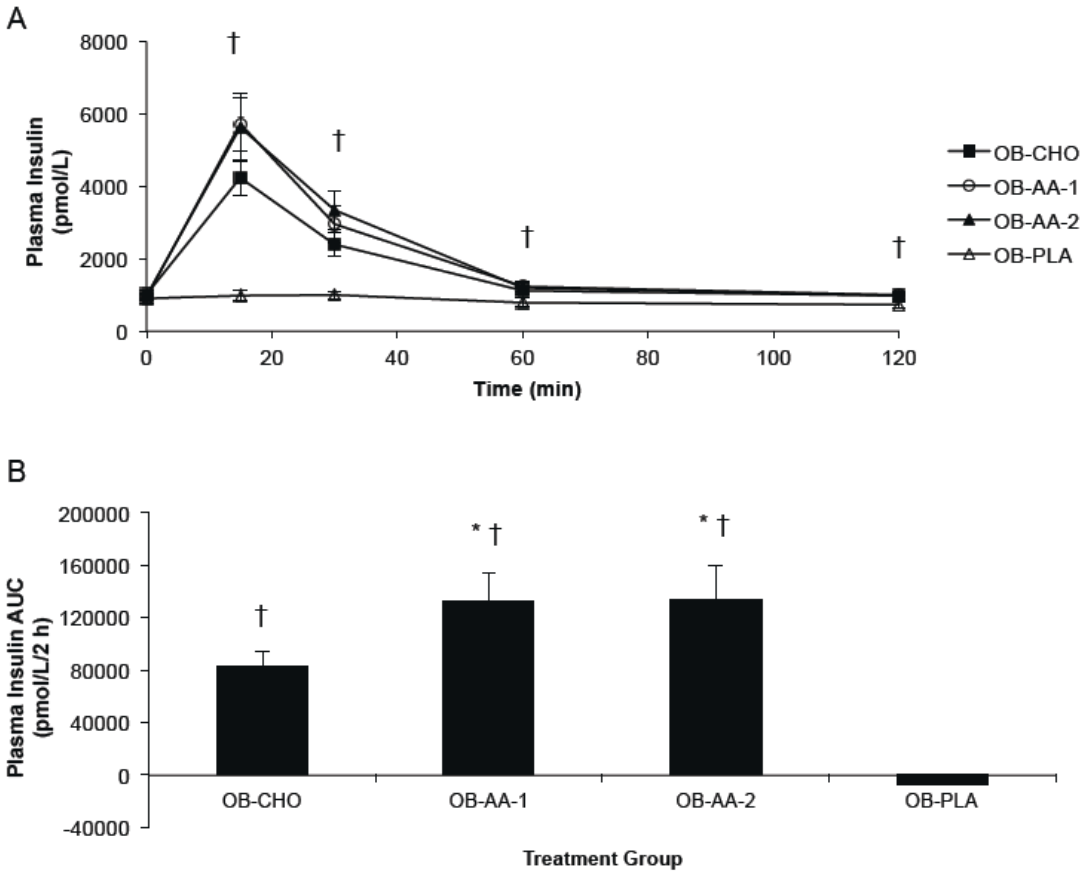

Figure 5.2. (A) Exp-1 plasma insulin during the OGTT. Values are means ± SE. †; $P < 0.05$ OB-PLA vs. all other treatments. (B) Exp-1 plasma insulin AUC during the OGTT. Values are means ± SE. *; $P < 0.05$ vs. OB-CHO. †; $P < 0.05$ vs. OB-PLA.

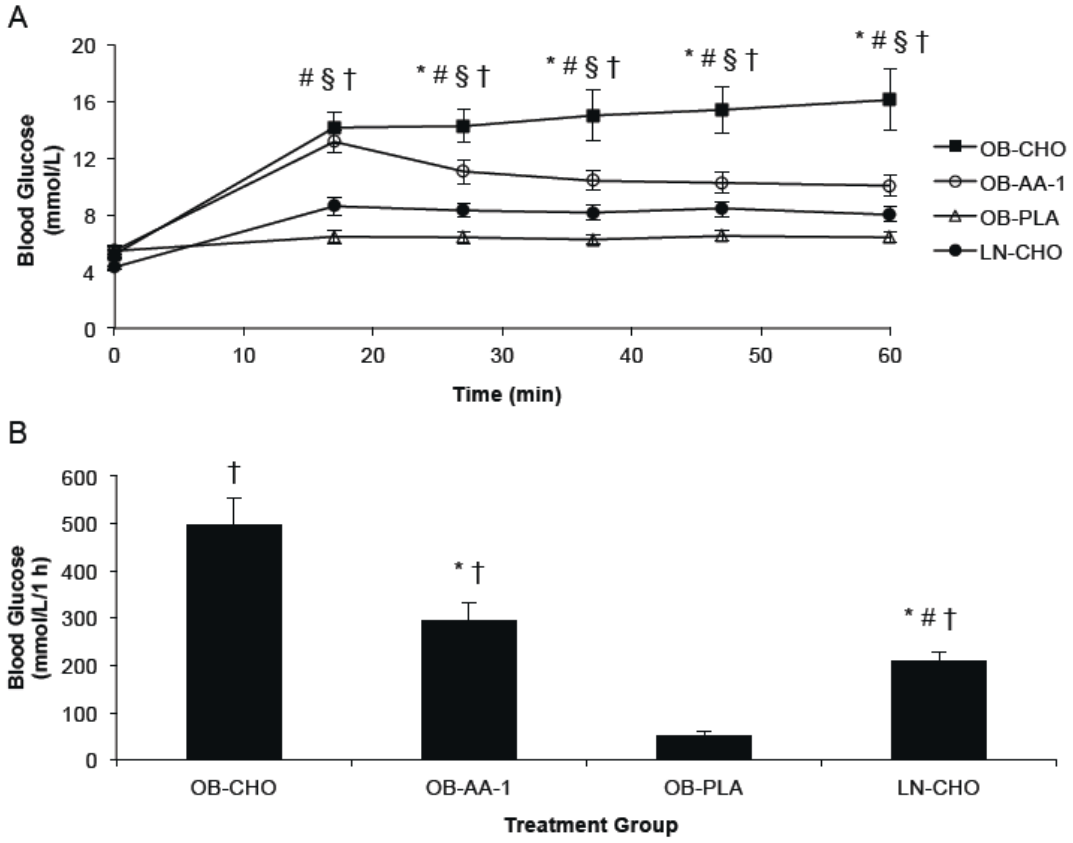

Figure 5.3. (A) Exp-2 blood glucose during the OGTT. Values are means ± SE. *, $P < 0.05$ OB-CHO vs. OB-AA-1; #, $P < 0.05$ OB-CHO vs. LN-CHO; §, OB-AA-1 vs. LN-CHO; †, $P < 0.05$ OB-PLA vs. all other treatments. (B) Exp-2 blood glucose AUC during the OGTT in Zucker rats. Values are means ± SE. *, $P < 0.05$ vs. OB-CHO; #, vs. OB-AA-1 †, $P < 0.05$ OB-PLA.

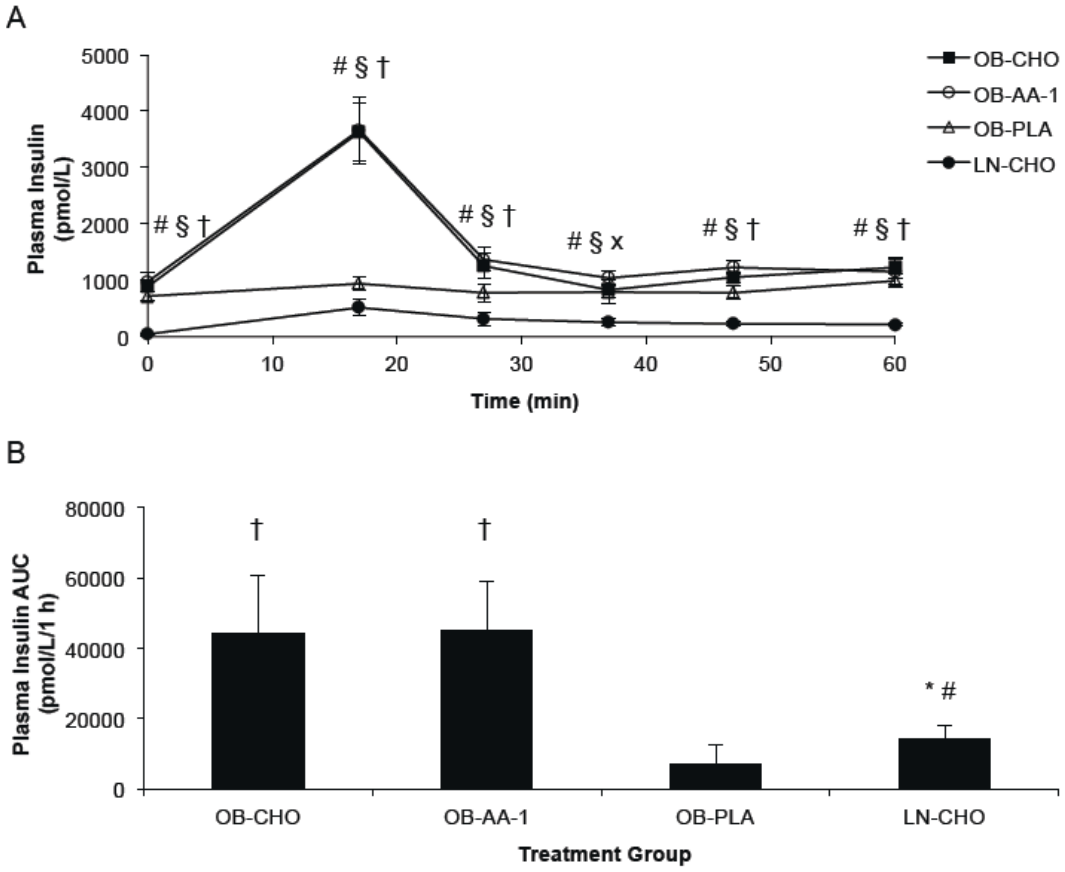

Figure 5.4. (A) Exp-2 Plasma insulin during the OGTT in Zucker rats. Values are means ± SE. *, $P < 0.05$ OB-CHO vs. OB-AA-1; $^{\#}$, $P < 0.05$ OB-CHO vs. LN-CHO; §, OB-AA-1 vs. LN-CHO; $^{\times}$, $P < 0.05$ OB-PLA vs LN-CHO †, $P < 0.05$ OB-PLA vs. all other treatments. (B) Exp-2 plasma insulin during the OGTT in Zucker rats. Values are means ± SE. *, $P < 0.05$ vs. OB-CHO; #, vs. OB-AA-1 †, $P < 0.05$ OB-PLA.

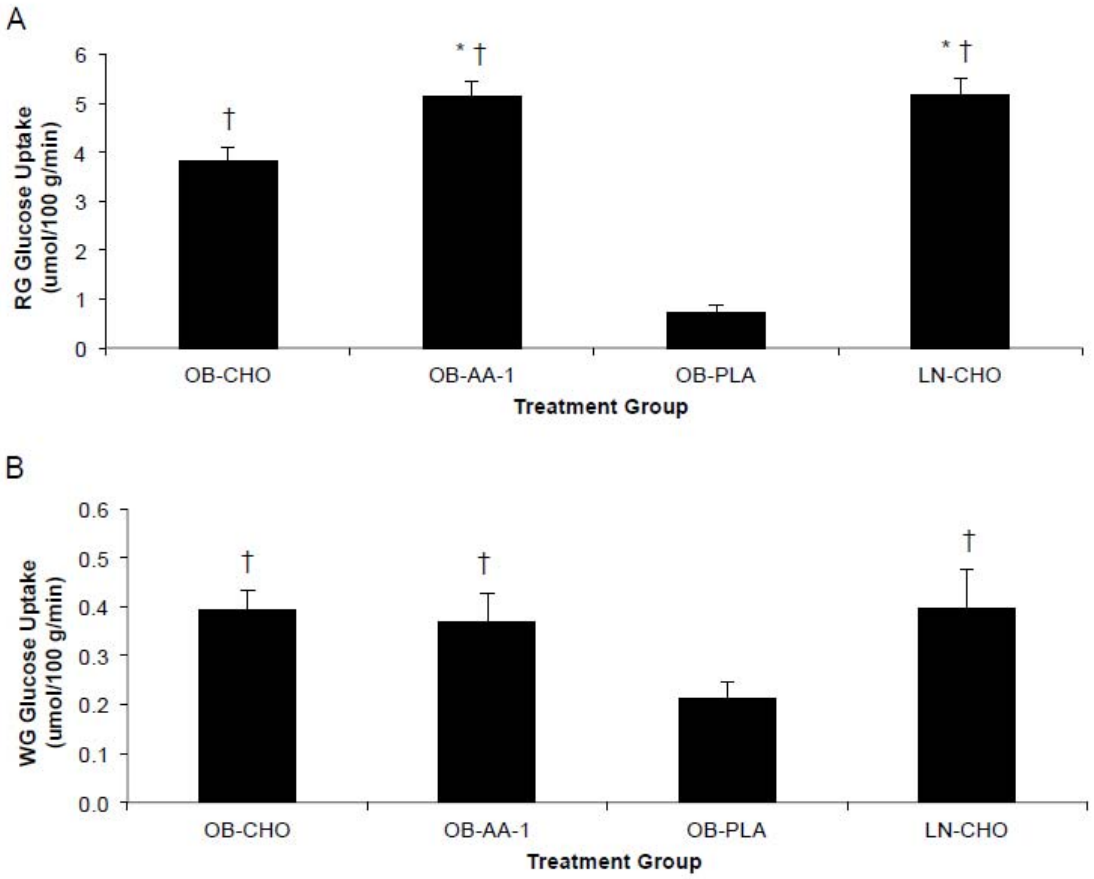

Figure 5.5. Exp-2 glucose uptake in the (A) red gastrocnemius and (B) white gastrocnemius during the OGTT in Zucker rats. Values are means $\pm$ SE. *, $P < 0.05$ vs. OB-CHO;†, $P < 0.05$ OB-PLA.

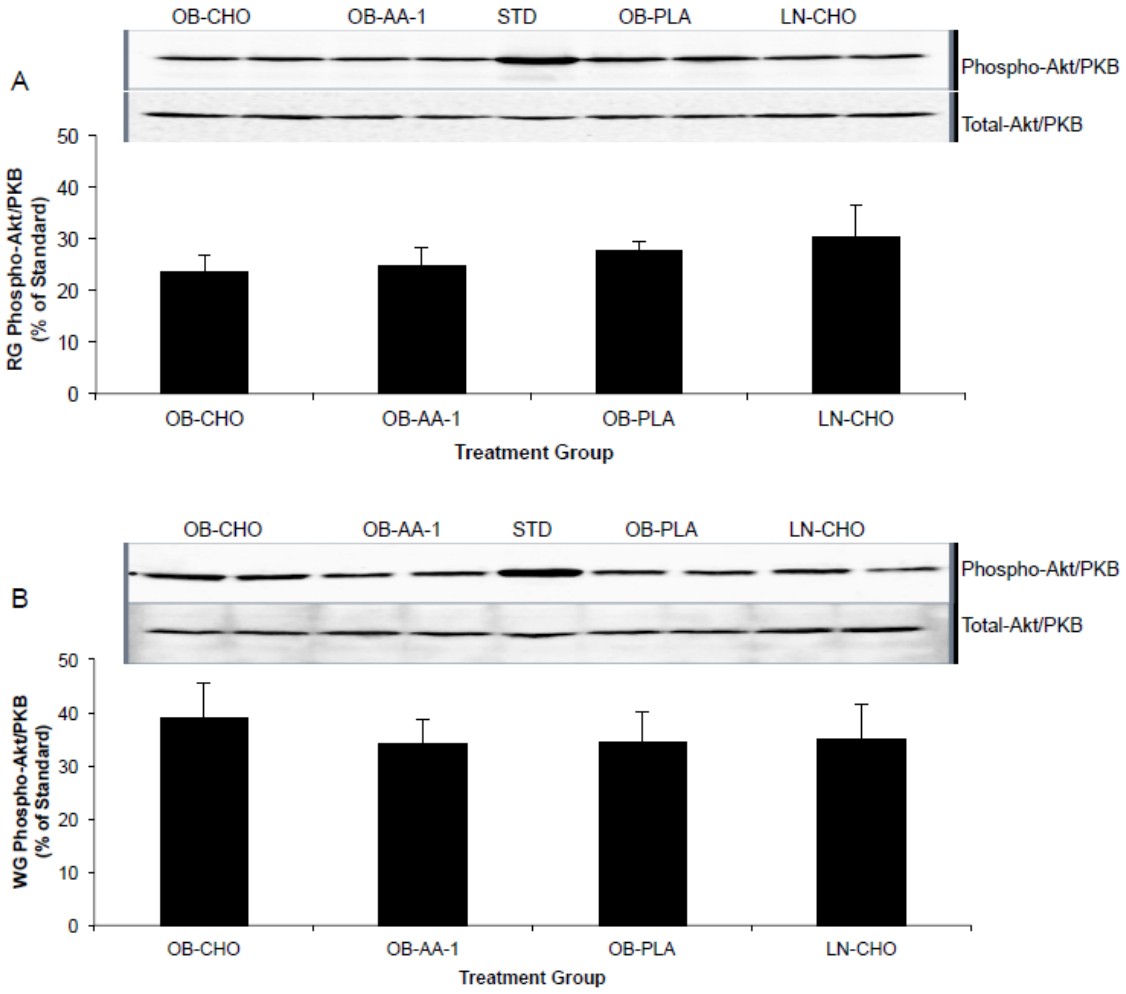

Figure 5.6. Exp-2 Akt Thr-308 phosphorylation following the OGTT in Zucker rats from the (A) red gastrocnemius and (B) white gastrocnemius muscles. Values are means ± SE.

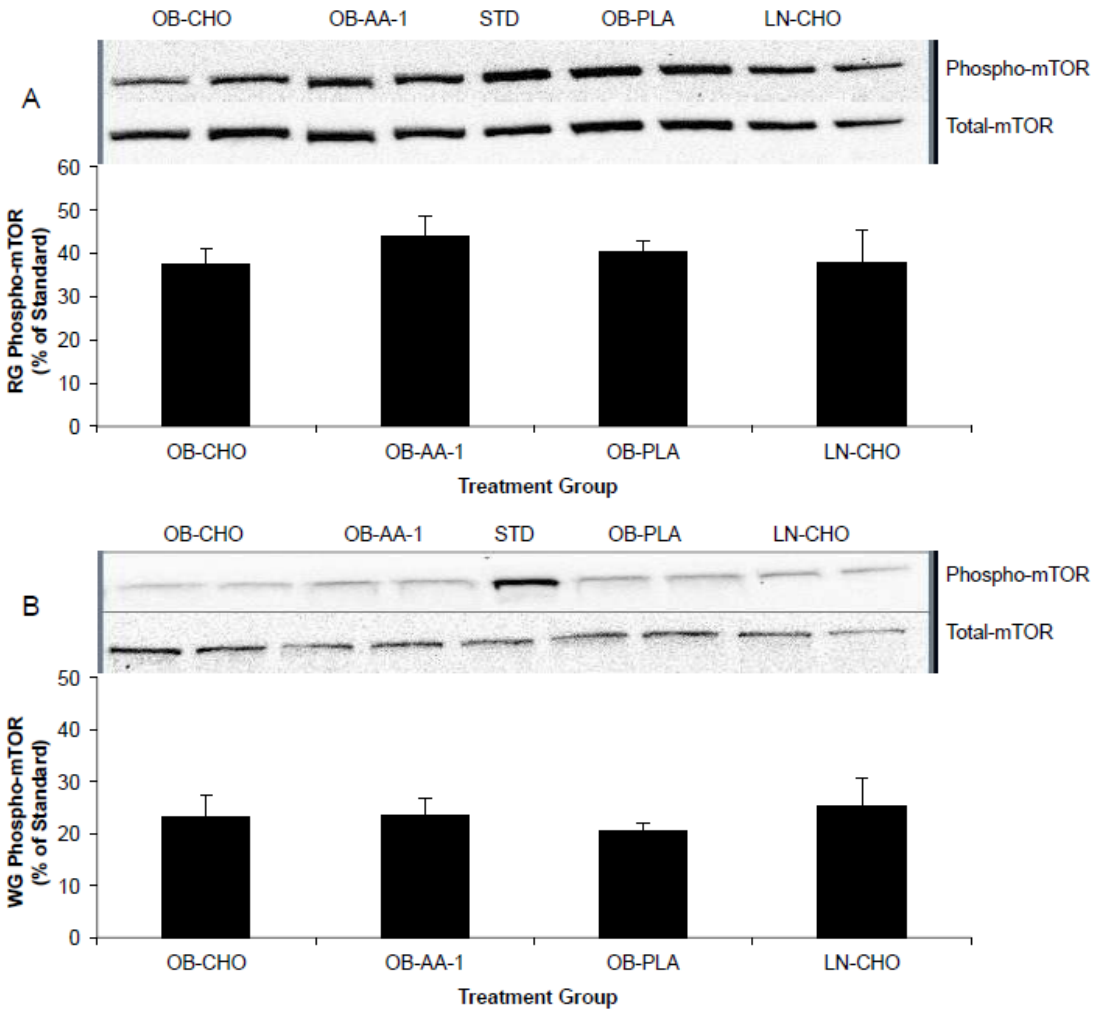

Figure 5.7. Exp-2 mTOR Ser-2448 phosphorylation following the OGTT in Zucker rats from the (A) red gastrocnemius and (B) white gastrocnemius muscles. Values are means ± SE.

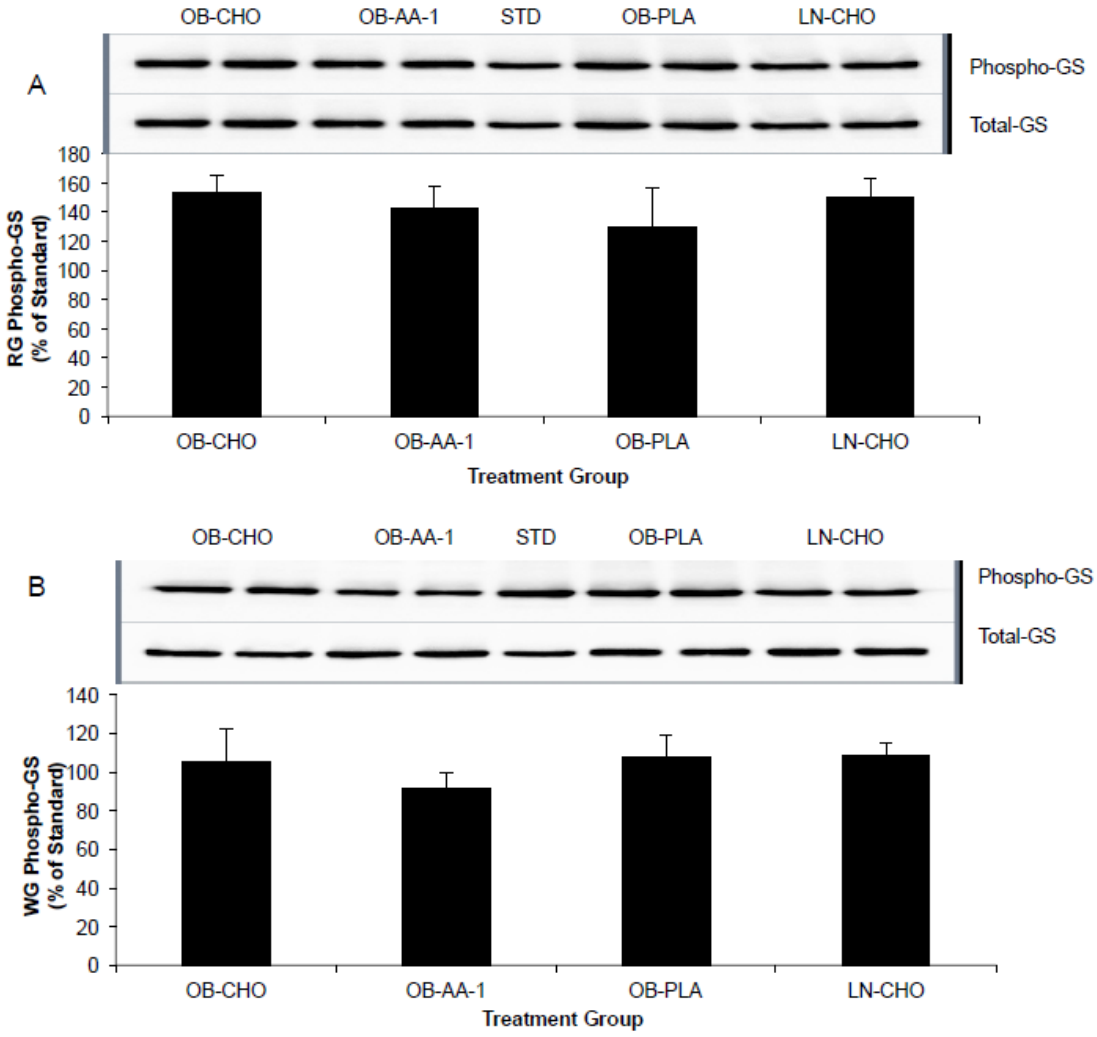

Figure 5.8. Exp-2 glycogen synthase Ser-641 phosphorylation following the OGTT in Zucker rats from the (A) red gastrocnemius and (B) white gastrocnemius muscles. Values are means ± SE. Values are means ± SE.

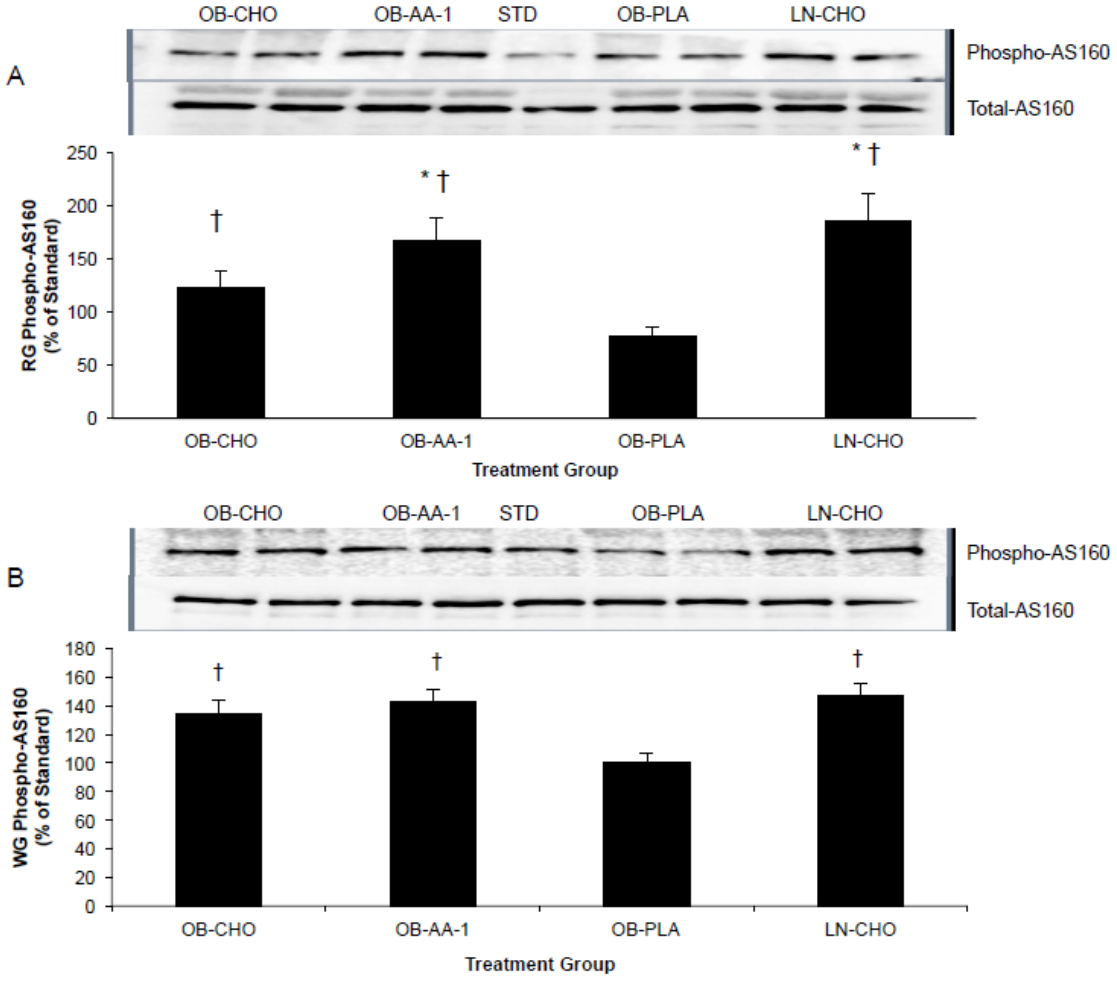

Figure 5.9. Exp-2 AS160 Thr-642 phosphorylation following the OGTT in Zucker rats from the (A) red gastrocnemius and (B) white gastrocnemius muscles. Values are means ± SE. *, $P < 0.04$ vs. OB-CHO; †, $P < 0.05$ OB-PLA.

Chapter VI: Detailed Materials Methods

EXPERIMENTAL ANIMALS AND ANIMAL CARE

Sprague Dawley and Zucker rats were obtained for the completion of these series of studies. The Sprague Dawley rats were chosen because they are one of the most commonly used outbred rat strains in medical research. These are considered very general rats, known for there ease of handling and a homogenous genetic background that yields consistent results. Zucker rats were selected because these rats are the most commonly used rodent model for obesity and insulin resistance research. The obese and their lean Zucker littermates are a substrain rat species characterized by a defective leptin receptor in the brain. Leptin is a molecule that binds to specific receptors in the hypothalamus and controls satiety. Under normal conditions, such as in the lean Zucker rat leptin will bind to its receptor causing the rat to feel full and stop eating. In contrast, obese Zucker rats have defective leptin receptors in which leptin cannot bind to its receptor leading to the rat continuously eating because it does no feel full. The excess caloric intake leads to the rapid development of obesity and insulin resistance.

Upon arrival at the University of Texas Animal Resource Center (ARC) rats were transported to the Bellmont Hall Animal Facility. Rats were housed individually and provided standard laboratory chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21° C and an artificial 12:12 h light-dark cycle was set. A reverse light cycle was used so rats would be tested during the dark cycle, which corresponds to their most active time. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conformed to the

guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Study 1 Experimental Design. For Study 1 Experiment-1 (Exp-1), 24 male Sprague Dawley rats approximately 7 weeks old were obtained from Harlan (Indianapolis, IN). Rats were randomly assigned to 1 of 4 groups: carbohydrate (CHO, n = 6), carbohydrate plus a 5-amino acid mixture (CHO-AA-1, n = 6), carbohydrate plus a 5-amino acid mixture with increased leucine concentration (CHO-AA-2, n = 6) or placebo (PLA, n = 6). These rats were used for the 2 h oral glucose tolerance test (OGTT). A second group of rats, 12 male Sprague Dawley rats approximately 12 weeks old were obtained from the ARC for Study 1 Exp-2. These rats were used for the determination of glucose uptake and for tissue analysis. The rats were randomly assigned to either carbohydrate (CHO, n = 6) or carbohydrate plus a 5-amino acid mixture (CHO-AA-1, n = 6). Following 1 week of acclimation, all rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior to the testing to familiarize them with the experimental procedures.

Study 2 Experimental Design. For Study 3, 29 male Sprague Dawley rats approximately 7 weeks old were obtained from Harlan. Upon arrival rats were randomly assigned to 1 of 4 groups: amino acid with submaximal insulin (AA-sINS, n = 9), submaximal insulin (sINS, n = 7), amino acid with no insulin (AA, n = 7) or basal (BAS, n = 6). Following 1 week of acclimation, all rats were handled daily to familiarize them with the experimental procedures.

Study 3 Experimental Design. For Study 2 Exp-1, 35 female obese Zucker rats approximately 7 weeks old were obtained from Charles River (Wilmington, MA). The obese Zucker rats were randomly assigned to 1 of 4 groups: obese-carbohydrate (OB-CHO, n = 9), obese-carbohydrate plus a 5 amino acid mixture (OB-AA-1, n = 9), obese-

carbohydrate plus a 5 amino acid mixture with increased leucine concentration (OB-AA-2, n = 9) or obese-placebo (OB-PLA, n = 8). These rats were used for the 2 h OGTT. A second group of rats, 18 female obese and 6 lean Zucker rats approximately 7 weeks old were obtained from Charles River for Study 2 Exp-2. These rats were used for the determination of glucose uptake and for tissue analysis. The rats were randomly assigned to either obese-carbohydrate (OB-CHO, n = 6), obese-carbohydrate plus a 5 amino acid mixture (OB-AA-1, n = 7) or obese-placebo (OB-PLA, n = 5). Lean Zucker rats were assigned to the carbohydrate group (LN-CHO, n = 6). Following 1 week of acclimation, all rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior to the testing to familiarize them with the experimental procedures.

ORAL GLUCOSE TOLERANCE TEST

An oral glucose tolerance test (OGTT) was performed on rats for both Study 1 and Study 3. For the Sprague Dawley rats in Study 1 Exp-1, following the familiarization period and after a 12 h fast, each rat was wrapped in a towel and the tip of their tail cut and bled. Rats were orally gavaged (8 ml/kg body weight) with 1 of 4 solutions: 1) CHO (22.5% glucose), 2) CHO-AA-1 (amino acid mixture in 22.5% glucose), 3) CHO-AA-2 (amino acid mixture with increased leucine concentration in 22.5% glucose) or 4) PLA (distilled water). For the obese Zucker rats in Study 2 Exp-1 following the familiarization period and after a 12 h fast, each rat was wrapped in a towel and the tip of their tail cut and bled. Rats were orally gavaged (8 ml/kg body weight) with 1 of 4 solutions: 1) OB-CHO (22.5% glucose), 2) OB-CHO-AA-1 (amino acid mixture in 22.5% glucose), 3) OB-CHO-AA-2 (amino acid mixture with increased leucine concentration in 22.5% glucose) or 4) OB-PLA (distilled water).

The amino acid mixture and OGTT used in Exp-1 was similar for Study 1 and Study 3. The amino acid mixture contained 5.28 mg cysteine, 3.36 mg methionine, 6.68 mg valine, 944.8 mg isoleucine and 6.68 mg leucine per 50 ml solution. The leucine concentration was increased to 50 mg/50 ml for the CHO-AA-2 treatment. Blood (0.5 ml) was taken from the tail immediately before the gavage and 15, 30, 60 and 120 min after. Blood was collected in one test tube containing EDTA (24 mg/ml, pH 7.4), and 0.1 ml of the EDTA blood sample was transferred to another test tube containing 10% perchloric acid (PCA). These tubes were stored at -80° C for later analysis. Immediately after the 120 min blood sample, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75.0 mg/kg body weight) at which time the red and white gastrocnemius were excised, freeze clamped in liquid nitrogen and stored at -80° C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight).

For Study 1 and Study 2 Exp-2, an OGTT was performed once again but with some modifications which allowed for the determination of skeletal muscle glucose uptake. For the Sprague Dawley rats in Study 1 Exp-1, following the familiarization period and after a 12 h fast, each rat was wrapped in a towel and the tip of their tail cut and bled. The rats were then orally gavaged (8 ml/kg body weight) with either CHO (22.5% glucose) or CHO-AA-1 (amino acid mixture in 22.5% glucose). The amino acid mixture was described previously in Exp-1. Fifteen min after the gavage, a bolus containing 40 μCi/kg body weight [^{3}H] 2-deoxyglucose (2-DG) and 20 μCi/kg body weight [U-^{14}C] mannitol was injected. Blood samples were collected before gavage, and at 2, 10, 20 and 45 min after the radioactive tracers were infused. Immediately after the 45 min blood sample, corresponding to 60 min after gavage, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight) at

which time the red and white gastrocnemius were excised, freeze clamped with tongs cooled in liquid nitrogen and stored at -80° C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight).

JUGULAR VEIN CATHETERIZATION

For the obese and leans Zucker rats in Study 3 Exp-2, all rats had a jugular vein catheter surgically implanted four days before the OGTT. Rats were placed on a heating pad covered with an underpad and subjected to isoflurane gas anesthesia for approximately 45 minutes in order to perform the jugular vein catheterization. The isoflurane (1.5%) was delivered from a vaporizer set at 1 L/min via a nose cone. The right jugular vein was accessed just above the clavicle. The hair over this site was first removed with hair clippers and cleaned with iodine. A small incision was made over the ventral thorax and immediately over the jugular vein. The vein was then cleared from the surrounding tissue until a small section of the vein could be visualized. A silk ligature was secured to the cranial portion of the vein to prevent blood flow going to the heart. Next, a small incision was made in the vein just distal to the cranial ligature. A 6 in thin walled catheter containing heparinized saline was carefully inserted into the vein and guided about 2 cm as to not enter the right atrium. A silk ligature was then tied around the inserted catheter to secure it in place. Next, the hair on the head and between the ears of the rat was removed with hair clippers, and cleaned with iodine, where a small incision was made. A hemostat was used to make a subcutaneous tunnel leading from the incision on the head to that over the thorax. The hemostat was then used to grasp the catheter and guide it back through the incision on the head of the rat. To ensure that the catheter was in the proper position a small amount of blood was drawn, flushed and heparinized saline stored in the catheter to prevent clotting. The incision sites over the thorax and head

were then sutured, providing both internal and external protection for the catheter. A small amount of Neosporin was applied along the surgery site during closure of the incisions to prevent infection. The catheter was sealed with a silicone patch and super glue. In order to prevent the rat from accessing the catheter a small strip of Velcro with a hole in it was sutured over the incision behind the head. The catheter was then threaded through the Velcro and the remaining 3-4 inches of the catheter coiled and placed between 2 Velcro strips (1). The rat was then removed from the isoflurane gas anesthesia, returned to a clean cage and placed on a heating pad. The rat remained under the supervision of the surgeon for approximately 30 min, or until it was able to right itself and move about its cage. After the rat had recovered from the anesthesia, post operative pain was controlled with a subcutaneous injection of carprofen (5 mg/kg body weight).

Each morning leading up to the experimental day the rats had their catheter flushed to maintain catheter patency and gavaged with saline to familiarize them with the OGTT procedure. Four days post surgery, and following a 12 hour fast, the rats were subjected to an OGTT. The rats were then orally gavaged (8 ml/kg body weight) with either 22.5% glucose (OB-CHO and LN-PLA), an amino acid mixture in 22.5% glucose (OB-AA-1) or distilled water (OB-PLA). The amino acid mixture was described previously in Exp-1. Fifteen min after the gavage, a bolus containing 40 μCi/kg body weight [^{3}H] 2-deoxyglucose (2-DG) and 20 μCi/kg body weight [U-^{14}C] mannitol was injected by syringe via the jugular vein catheter. The tail was bled and blood collected as describe above at 2, 10, 20 and 45 min after the radioactive tracers were infused for later analysis. Immediately after the 45 min blood sample, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight) at which time the red and white gastrocnemius were excised, freeze clamped in liquid nitrogen and

stored at -80° C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg body weight).

PERFUSATE PREPARATION

All perfusates for the hindlimb perfusion technique consisted of 6% bovine serum albumin (BSA) in KHB. First, a 23% BSA solution was made in KHB. The dissolved BSA was then sealed in dialysis tubing and allowed to dialyze for 48 h in 15 L KHB at 4° C. After 48 h the BSA concentration was check using a refractometer and the appropriate volume of KHB added to bring the BSA concentration to 8%. The BSA was filtered two times through fiberglass filter paper using a vacuum pump. The BSA concentration was checked again with a refractometer and then adjusted to 6% (pH 7.4). The 6% BSA solution was separated into 2 parts for the washout and perfusate. The washout contained 2 mM pyruvte as a cellular energy source. The perfusate contained 2 mM pyruvate, 6 mM glucose, 2 mM mannitol, 0.2 μCi/ml 2-[^{3}H] deoxyglucose (2-DG) and 0.15 μCi/ml [^{14}C(U)]-sucrose (^{14}C-sucrose). For the insulin-stimulated treatment groups (AA-sINS and sINS) 200 μU/ml insulin was added to both the washout buffer and the perfusate. For the amino acid treatment groups (AA-sINS and AA) 2 mM of an amino acid mixture was also added to both the washout buffer and the perfusate. The amino acid mixture contained 5.28 mg cysteine, 3.36 mg methionine, 6.68 mg valine, 944.8 mg isoleucine and 6.68 mg leucine per 50 ml solution.

HINDLIMB PERFUSION

For Study 2, rats were subjected to the hindlimb perfusion technique. The purpose of the surgical preparation for this technique is to direct perfusate flow to the iliac artery, through the capillary bed of the hindlimb and out of the abdominal section of the inferior vena cava. This was done by ligating all the major vessels branching from

the abdominal aorta and vena cava, except for the common iliac artery, iliac vein and the great vessels. Only the right hindblimb was perfused during the equilibrium and tracing periods. Following a 12 h fast, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight). Rats were then placed on a heating pad covered with an underpad in order to maintain normal body temperature and circulation throughout the surgery. A ligature was first tied around the tail. A midline abdominal incision was then made through the skin and abdominal wall from the pubic symphysis to the ziphoid process. The superficial epigastric arteries leading the skin were exposed by retracting the skin, the vessels then ligated with sutures. The stomach, spleen, pancreas and intestinal tract were removed from the abdominal cavity and wrapped in parafilm allowing access to the inferior vena cava and descending aorta. The hypogastric arteries on the inside of the abdominal wall were then ligated. The bladder, testis and inferior mesenteric arteries were also ligated. The descending colon was ligated just inferior to the transvers colon and just superior to the rectum, then excised. Using dampened gauze, adipose tissue and any remaining fascia in the abdominal cavity anterior to the aorta and vena cava was removed. The iliolumbar arteries and veins and pubic-epigastric truck were also ligated.

Ligatures were then placed loosely around the aorta and vena cava inferior to the renal vessels and around the vena cava superior to the iliolumbar vessels. A igature was placed around the left iliac artery and tightened to prevent perfusate flow to the left hindlimb. The ligature around the descending aorta was tightly secured and a small incision was made. A polyethylene catheter was inserted and threaded to the tip of the femoral artery. Saline solution was pushed through the catheter from a 10 ml syringe. The superior ligature around the inferior vena cava was then tightened causing the vessel to distend. The venous catheter was then inserted and positioned below the distal

ligature. The distal ligature was then tied in order to secure the catheter. Approximately 10 ml of saline was pushed through the arterial catheter, flushing the right hindlimb and to prevent clotting. During the infusion of saline solution the rat was euthanized with an intracardiac injection of sodium pentobarbital (65 mg/kg body weight). After the infusion the catheters were placed in line with the hindlimb perfusion apparatus. The right hindlimb was washed out with Krebs-Henseleit buffer (KHB) at a flow rate of 6 ml/min. Following the 10 min washout period the arterial line was switch to the perfusate and set at a flow rate of 4 ml/min. The perfusions were performed at 37° C and continued for a total of 25 minutes, at which time the right gastrocnemius excised, freeze clamped in liquid nitrogen, and stored at - 80°C for later analysis.

The perfusion system consisted of an arterial reservoir, roller pump, lung, water jacket, bubble trap and effluent reservoir enclosed in a Plexiglas chamber accessible from the front. The perfusate was mixed continuously on a magnetic stir plate. The perfusate was drawn from the arterial reservoir and passed through a roller pump. The perfusate then passed through an artificial lung and gassed with a mixture of 95% O_2 and 5% CO_2. The perfusate then passed through a heated water jacket maintained at 37° C, and then through a bubble trap before entering the hindlimb of the rat via the arterial catheter. The bubble trap was equipped with a manometer to measure the arterial pressure. After circulating through the right hindlimb, the perfusate exited through the venous catheter and dripped into the effluent reservoir.

MUSCLE GLUCOSE UPTAKE

Rates of 2-DG uptake were determined in both red and white gastrocnemius muscle samples for Study 1 Exp-2 and Study 3 Exp-2 and in mixed gastrocnemius muscle samples in Study 2. 2-DG is a non-metabolized glucose analogue that has similar

rates of removal from the blood into the muscle tissue as glucose under a physiological range of insulin concentrations. The amount of tracer contained within the extracellular space was determined by the amount of ^{14}C mannitol (^{14}C sucrose for Study 3) retained in the tissue. Mannitol and sucrose are sugars that are not transported by glucose transporters and therefore occupy only the extracellular space. Approximately 80-100 mg of muscle were dissolved in 1 ml of 1 N potassium hydroxide (KOH) by incubating for 15 min at 65° C, vortexed then incubated for an additional 5 min at the same temperature. Next, an equal volume of 1 N hydrochloric acid (HCl) was added to the digested samples and vortexed to neutralize the samples. To determine the specific activity of the blood, a 200 µl aliquot of the PCA extract was added to 1 ml of 1 N KOH, then neutralized with an equal volume of 1 N HCL. A 300 µl aliquot of neutralized muscle and blood samples were then added to a vial containing 6 ml Bio-Safe II counting cocktail (Research Products International, Mount Prospect, IL). Duplicate samples were counted in a liquid scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA) preset for simultaneous counting of [^{3}H] and [^{14}C] DPM. Quenching was determined by counting prepared standards. The accumulation of intracellular 2-DG was indicative of muscle glucose uptake. For Study 1 Exp-2 and Study 3 Exp-2, the specific activity of the blood for [^{3}H] and [^{14}C] was determined using the integral of the plasma 2-DG and [U-^{14}C] mannitol over the 15-60 min per glucose molecule. The specific activity of the perfusate for [^{3}H] and [^{14}C] in Study 2 was determined by calculating the perfusate 2-DG and [U-^{14}C] mannitol per glucose molecule. The extracellular space was calculated using the total muscle ^{14}C DPM and its specific activity. The intramuscular accumulation of 2-DG was calculated by subtracting its extracellular space DPM from its total muscle DPM divided by its specific activity.

BLOOD ANALYSIS

A drop of blood was used to measure blood glucose with a portable glucose analyzer (One Touch Ultra 2; LifeScan Inc., Milpitas, CA). The system was calibrated according to the manufacturer's instructions. A drop of blood was placed on a reading strip inserted into the glucose analyzer. The reliability and validity of the system was verified using the Trinder assay (5).

Spun down EDTA samples were used for the determination of plasma insulin. . Plasma insulin was determined by a radioimmunoassay kit (Linco, St. Charles, MO) using a double antibody procedure. The ^{125}I-labeled insulin was added to tubes containing plasma samples, standards and quality controls. Rat insulin antiserum was then added to these tubes and incubated overnight at 4° C during which time the ^{125}I-labeled and unlabeled insulin competed for antibody binding. This antibody-antigen complex was then precipitated by the addition of a carrier and an antibody to the carrier. The bound and free ^{125}I-labeled insulin was separated by centrifugation. Standards were used to construct a standard curve and samples were plotted on this curve.

WESTERN BLOT ANALYSIS

Approximately 150 mg of muscle was homogenized (1:9) in an ice-cold buffer (pH 7.4) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylene glycol tetraacetic acid (EGTA), 50 mM sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM glycerolphosphate, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Benzamidine, and 0.5 mM sodium Vanadate (Na_3VO_4) with a glass tissue grinder pestle (Corning Life Sciences, Acton, MA). The homogenate was then centrifuged at 14,000 x g for 10 min at 4° C. Aliquots of the supernatant were stored

at -80° C for later analysis. The protein concentration of the homogenate was determined using the Lowry method (4).

The phosphorylation of protein kinase B (Akt/PKB), mammalian target of rapamycin (mTOR), Akt substrate of 160 kDa (AS160) and glycogen synthase (GS) were used as an indirect measurement of activity for Study 1 Exp-2, Study 2 and Study 3 Exp-2. Sample protein (100 μg for Akt/PKB, 70 μg mTOR, AS160 and 60 μg for GS) was combined with an equal amount (1:1) of Laemmli sample buffer (125 mM Tris, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.008% bromophenol blue, pH 6.8) (3) and boiled for 5 min. Next, sample proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins separated on either an 8% (mTOR and AS160) or 12% (Akt/PKB and GS) polyacrylamide resolving gel for either 1 h (Akt/PKB) or 1.5 h (mTOR, AS160 and GS). The resolved proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer unit and blocked in 7% nonfat dry milk in Tris-Tween-buffered saline (NFDM/TTTBS) for 1 h at room temperature. The membranes were then incubated with either affinity purified anti-phospho-Akt/PKB (Thr-308) (Cell Signaling Technology, Danvers, MA), anti-phospho-mTOR (Ser-2448) (Cell Signaling Technology), anti-phospho-AS160 (Thr-642) (Millipore, Billerica, MA) or anti-phospho-GS (Ser-641) (Cell Signaling Technology) overnight at 4° C. These phosphorylation sites were chosen as an indirect measurement of activity because they represent the primary sites of phosphorylation of the respective protein under insulin-stimulated conditions. The primary antibodies were diluted to either 1:500 (phospho-Akt/PKB), 1:800 (phospho-AS160) or 1:1000 (phospho-mTOR and phospho-GS) in TTBS containing 2% NFDM. Following the overnight incubation the membranes were washed for 3, 5-min washes in TTBS then incubated for either 1 h (Akt/PKB and GS) or 2 h (mTOR and AS160) at room temperature with the

species-specific (anti-rabbit) immunoglobulin G (IgG) secondary antibodies (Cell Signaling Technology). The secondary antibodies were diluted to either 1:750 (Akt/PKB and AS160), 1:900 (mTOR) or 1;2000 (GS) in TTBS containing 2% NFDM. The membranes were washed with 5, 8-min washes with TTBS and antibody binding was visualized by enhanced chemiluminescence (ECL) in accordance to the manufacturer's instructions (Perkin Elmer, Boston, MA). Images were captured by using a charge-coupled device camera in a ChemiDoc system (BioRad, Hercules, CA) and saved to a computer. Density of the bands were quantified with Quantity One analysis software (BioRad) and expressed as a percentage of a standard run on each gel.

After the phosphorylation status of each protein had been determined, the primary phosphorylated-antibody was stripped from the membrane to determine either the total protein concentration or alpha-tubulin to confirm that an equal amount of protein was loaded onto each gel. Membranes were placed in a stripping solution (100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris base (pH 6.7)) and heated at 60° C for 1 h. Membranes were washed for 3, 15-min washes with TTBS to remove the stripping solution. The primary and secondary antibody concentrations, incubation times, washing and quantification were the same as those described for the determination of phosphorylation status.

PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY

Both the insulin receptor substrate-1 (IRS-1) associated and total phosphatidylinositol 3-kinase (PI 3-kinase) activity was determined in mixed gastrocnemius muscle obtained from Study 2 using an enzyme-linked immunosorbent assay (ELISA) kit. For IRS-1 associated PI 3-kinase activity, approximately 150 mg of muscle was homogenized (1:9) in an ice-cold homogenization buffer as described above.

Aliquots of the supernatant were stored at -80° C for later analysis. The protein concentration of the homogenate was determined using the Lowry method (4).

One milligram of sample protein were immunoprecipitated with 8 μg of anti-IRS-1 (Millipore, Billerica, MA) and homogenization buffer overnight at 4° C. Protein-A Sepharose (PRO-A) beads were then prepared by washing once with phosphate triton azide (PTA) and twice with homogenization buffer and centrifuged at 14,000 x g for 5 min at 4° C between each wash. After the final wash, the packed PRO-A beads were resuspended in homogenization buffer at a 1:1 dilution. One hundred microliters of PRO-A slurry was added to the anti-IRS-1 immunoprecipitates for 1.5 h at 4° C with rotation. Following the incubation, samples were centrifuged at 14,000 x g for 10 min at 4° C. The immunocomplex was then washed successively with the following: Buffer A (10% octylphenoxypolyethoxyethanol (IGEPAL), 100 mM Na_3VO_4, 1 M DTT, phosphate buffered saline (PBS)), Buffer B (1 mol/L Tris-HCl (pH 7.5), 2 M lithium chloride ($LiCl_2$), 100 mM Na_3VO_4, 1 M DTT), and Buffer C (1 M Tris-HCl (pH 7.5), 5 M sodium chloride (NaCl), 100 mM Na_3VO_4, 10 mM EDTA, 1 M DTT). Washing was performed once in buffers A and B and twice in buffer C. The packed beads were then diluted 1:1 in 5 x KBZ buffer (provided in kit), 1 M DTT, 10 mM adenosine-5'-triphosphate (ATP) and 100 μM $PI(4,5)P_2$ substrate. The substrate was incubated for 4 h and the product $PI(3,4,5)P_3$ and was detected by an ELISA kit (catalogue# K1000s, Echelon Biosciences Inc., Salt Lake City, UT). The amount of phosphatidylinositol (3,4,5)-triphosate (PIP3) produced by extracted PI 3-kinase from the sample was proportional to the PI 3-kinase activity.

For the determination of total PI 3-kinase activity approximately 150 mg of mixed gastrocnemius muscle was homogenized (1:9) in an ice-cold 0.5 M TCA. The homogenate was then centrifuged at 1,500 rpm for 5min at 4° C. The supernatant was

discarded and the pellet was resuspended in 3 ml of 5% TCA/1 mM EDTA. The protein concentration was then determined using the Lowry method (4). One milligram of protein was then subjected to the extraction protocol in accordance to the manufacturer's instructions. The extracted substrate was incubated for 1 h and the product PI(3,4,5)P_3 was detected by an ELISA kit (catalogue# K2500s, Echelon Biosciences Inc.). The amount of PIP3 produced by extracted PI 3-kinase from the sample was proportional to the total PI 3-kinase activity.

PLASMA MEMBRANE GLUCOSE TRANSPORTER 4 PROTEIN CONCENTRATION

The plasma membrane glucose transporter 4 (GLUT4) protein concentration was determined in mixed gastrocnemius muscle samples obtained from Study 2. Approximately 150 mg of muscle sample was homogenized in an ice cold buffer (8 x wt/vol) containing 20 mM HEPES (pH 7.2), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na_3VO_4, 10% glycerol, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin A and 1 mM PMSF. The homogenate was centrifuged at 33,000 rpm for 30 min at 4° C and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in an ice-cold buffer (4 x wt/vol) in which 1% Trition X was added. The resuspended pellet was then centrifuged at 11,000 rpm for 10 min at 4° C. The supernatant, representing the plasma membrane, was collected.

To ensure that purified plasma membrane fractions were being used for analysis the enzymatic activity of the plasma membrane marker 5'-nucleotidase in the plasma membrane was compared to its activity in the cytosolic fraction according to the manufactures instructions based on the method developed by Fiske and SubbaRow (2). Ten microliters of the plasma membrane or cytosolic fractions were added to 200 ul of a reaction cocktail containing 0.5 M glycine, 0.01 M magnesium chloride ($MgCl_2$) and

0.005 M adenosime monophosphate (AMP). The reaction was run for 45 min at which time 400 µl of 8% trichloroacetic acid (TCA) was used to stop the reaction. Samples were then centrifuged at 2500 rpm for 10 min and 200 µl of the supernatant was aliquoted to a test tube containing acid molybdate and water. The tubes were mixed by gently vortexing and then 25 µl of Fiske SubbaRow cocktail was added. Following a 10 min incubation period the absorbance was read at 660 nm using spectrophotometer.

The plasma membrane GLUT4 protein concentration was determined in the presence and absence of a submaximal insulin concentration. The protein concentration for the plasma membrane and crude homogenate was determined using the Lowry method (4). Sample protein (40 µg) was subjected to SDS-PAGE and the protein was separated on a 12% resolving gel. Next, the resolved proteins were transferred to a PVDF membrane using a semidry transfer unit and blocked in NFDM-TTBS for 1 h at room temperature. Using a visible molecular weight marker (Bio-Rad) as a guide the PVDF membranes were cut into an upper and lower membrane section. The upper membrane section was probed with affinity purified anti-sodium-potassium-ATPase (Na^+K^+-ATPase) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4° C. The Na^+K^+-ATPase pump is a ubiquitous plasma membrane marker used to demonstrate equal protein loading across samples. The lower membrane section was probed with affinity purified anti-GLUT4 antibody (donated by Dr. Samuel Cushman, National Institute of Diabetes and Digestive Kidney Disease, Bethesda, MD) overnight at 4° C. Following 3, 5-min washes in TTBS the membrane was incubated for 1 h at room temperature with the species-specific immunoglobulin G secondary antibody. The membrane was then washed with 3, 5-min washes in TTBS and antibody binding was visualized by ECL in accordance to the manfacturer's instructions (Perkin Elmer). Images were captured by using a charge-coupled device camera in a ChemiDoc system

(BioRad) and saved to a computer. The density of each band was quantified using Quantity One analysis software (BioRad) and expressed as a percentage of an insulin-stimulated standard run on each gel.

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Chapter VII: Summary

Recent investigations have demonstrated that amino acids, specifically the branch-chained amino acid, are involved in blood glucose regulation. Of the branchd-chain amino acids, two of them, isoleucine and leucine, have been reported to have direct actions on blood glucose levels. Several studies have shown that both of these branched-chain amino acids may acutely lower circulating blood glucose levels (1, 2, 6) in healthy tissue. But the ability of amino acids to lower the blood glucose response holds great clinical significance for individuals afflicted by insulin resistance. Many questions remain, however, as to how amino acids exert their affect on blood glucose levels. Several lines of questioning that have been explored in this dissertation, as well as in other laboratories, are the roles of insulin secretion, cellular signaling and skeletal muscle glucose uptake.

It is well established that consuming carbohydrates with either protein (7, 10) or amino acids (8, 9) synergistically enhance insulin secretion. The attenuated blood glucose response to these treatments has been attributed to greater insulin secretion (4, 8, 10). However, recent studies have reported that both leucine and isoleucine enhanced skeletal muscle glucose uptake in the absence of insulin (1, 6) *in vitro*. These findings suggest a minor role for insulin in amino acid-induced glucose clearance. Therefore, improved skeletal muscle glucose clearance, and alterations to its underlying signaling pathways may explain the enhanced glucose response.

Some investigators have begun to explore the significance of cell signaling in amino acid-induced glucose clearance *in vitro*. Both leucine and isoleucine have been found to increase skeletal muscle glucose uptake in the absence of insulin *in vitro* (1, 2,

6). Nishitani et al. (6) reported that both phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase C (PKC) were involved in leucine-induced glucose uptake across isolated soleus muscle as uptake was significantly reduced when the respective specific inhibitors for each kinase was introduced to the incubation medium. In contrast, pre-treatment with rapamycin, a specific inhibitor of the mammalian target of rapamycin (mTOR) had no effect. In agreement, Doi et al. (1) reported that isoleucine-induced glucose uptake in C_2C_{12} myotubes was inhibited in the presence of a specific inhibitor of PI 3-kinase but not in the presence of an inhibitor of mTOR, suggesting that PI 3-kinase signaling, rather than mTOR signaling, may be involved in amino acid-induced glucose uptake.

The *in vitro* results support the involvement of PI 3-kinase signaling in amino acid-induced muscle glucose uptake. However, there has not been a comprehensive investigation of the *in vivo* impact of amino acid ingestion on glucose homeostasis. Furthermore, despite the lack of evidence for a direct link of mTOR to glucose uptake, a potential role for mTOR warrants further investigation as this protein is activated by amino acids and may be involved in glycogen synthesis. Thus, the primary objective of these dissertation studies was to determine the effects of amino acid supplementation on glucose tolerance, insulin resistance, insulin secretion and cellular signaling.

STUDY 1

In Study 1 Experiment-1 (Exp-1), the primary aim was to determine if an amino acid mixture lowers the blood glucose response to an oral glucose challenge and if the potency of the mixture was enhanced by increasing the leucine concentration. Briefly, Sprague Dawley rats were orally gavaged (8ml/kg body weight) with one of four supplements: carbohydrate (CHO), carbohydrate plus a 5 amino acid mixture (CHO-AA-1, carbohydrate plus a 5 amino acid mixture with increased leucine concentration (CHO-

AA-2) or placebo (PLA). Blood was collected from the tail at 0, 15, 30, 60 and 120 min post supplementation. There were no significant differences in fasting blood glucose levels among the treatment groups. Following water supplementation for the PLA group, there were only minimal changes in blood glucose. The fact that there was little change in blood glucose response for the PLA group was crucial as it suggests that the rats were familiarized with the procedure for the appropriate amount of time and not under stress throughout the oral glucose tolerance test (OGTT). Blood glucose was significantly lowered compared to the other treatment groups post supplementation for PLA. The blood glucose response peaked at 15 min for CHO, CHO-AA-1 and CHO-AA-2, but was significantly lower for both CHO-AA-1 and CHO-AA-2 at 15, 30 and 60 min after supplementation. However, there was no statistical difference in blood glucose between CHO-AA-1 and CHO-AA-2 at any time point. The lower blood glucose response in the amino acid groups were further demonstrated when the area under the curve (AUC) was calculated. Since the blood glucose response was similar between CHO-AA-1 and CHO-AA-2, the carbohydrate plus amino acid mixture with the lower leucine concentration (CHO-AA-1) was used for the remainder of the Sprague Dawley OGTT studies.

Because insulin is the primary hormonal regulator of blood glucose, the plasma insulin response during the OGTT was determined to assess whether an increase in insulin secretion could explain the lower blood glucose response in the amino acid treated rats. Fasting insulin levels were similar between treatment groups and the PLA plasma insulin was significantly lower than the other treatment groups at 15 and 30 min post supplementation. There were no significant differences in the insulin response between CHO-AA-1 and CHO-AA-2 at 15, 30, 60 and 120 min post supplementation. Furthermore, insulin levels were similar between these treatment groups when the AUC was calculated.

Several labs have report that individual amino acids, especially leucine and isoleucine when administered alone, increase muscle glucose uptake *in vitro*. The evidence to date suggests that isoleucine is a more potent glucose lowering agent compared to leucine. This may begin to partially explain why we did not observe a lower glucose response when the concentration was increased for the CHO-AA-2 treatment. Results from Study 1 Exp-1 demonstrated that the blood glucose response was attenuated by the amino acid mixture, and that the improved glucose tolerance was not associated with an increase in insulin secretion. These findings also suggested that increasing the leucine concentration of the amino acid mixture does not provide additional improvements for the glucose and insulin responses.

In Study 1 Exp-2, the primary aim was to determine if the improved glucose tolerance observed in Study 1 Exp-1 was due to increased skeletal muscle glucose uptake and if differences in uptake were characterized by alterations in protein signaling. Briefly, Sprague Dawley rats were orally gavaged (8ml/kg body weight) with either carbohydrate (CHO) or carbohydrate plus the amino acid mixture (CHO-AA-1). Fifteen min after the gavage, a bolus containing a radiolabled glucose analogue was injected by syringe via a tail vein. The tail was bled and blood collected at 0, 17, 27, 37, 47 and 60 min post supplementation. Immediately after the 60 min blood draw, the rats were sacrificed and muscle excised from the hindlimb. The blood glucose response to the glucose challenge was reduced in the CHO-AA-1 treatment group compared to CHO. The difference in blood glucose was likely not due to alterations in insulin secretion as plasma insulin was similar between treatment groups.

Next, it was determined whether the lower blood glucose was due to enhanced amino acid-induced skeletal muscle glucose uptake. Rates of glucose uptake were significantly greater in both red and white gastrocnemius muscles for the CHO-AA-1

treatment group compared to CHO. Since glucose uptake was increased with no differences in the plasma insulin response between treatment groups, it raised the possiblity that cell signaling may play an important role in the amino acid-stimulated glucose clearance.

The increased glucose uptake observed with the amino acid mixture is presumably a result of increased plasma membrane glucose transporter 4 (GLUT4). GLUT4 protein concentration was not measured because the glucose transporter would likely have been recycled from the plasma membrane 1 h post supplementation. There was no difference in total protein concentration for Akt/PKB, mammalian target of rapamycin (mTOR), glycogen synthase (GS) or Akt substrate of 160 kdDa (AS160) to the acute ingestion of either the carbohydrate or amino acid mixture. However, the phosphorylation of mTOR, GS and AS160 were increased by the amino acid mixture in the red gastrocnemius. AS160 phosphorylation was increased in the white gastocnemius as well. Therefore, the lower glucose response for the amino acid treatment appeared due to a greater rate of blood glucose clearance and this response was associated with increased phosphorylation of intracellular signaling proteins rather than greater insulin secretion.

In conclusion, Study 1 demonstrated that the amino acid mixture attenuates the blood glucose response to an oral glucose challenge and this appears due to enhanced skeletal muscle glucose uptake and intracellular signaling. The fact that the amino acid mixture improved glucose tolerance in a rodent model in which glucose metabolism was already adequate was intriguing. The results from Study 1 clearly demonstrate the potential for the amino acid mixture to improve glucose tolerance in an insulin resistant rodent model.

STUDY 2

The primary aim of Study 2 was to determine if perfusing the amino acid mixture increased skeletal muscle insulin-stimulated and non-insulin-stimulate glucose uptake and if differences in glucose uptake were associated with alterations in PI 3-kinase activity, AS160 physorylation and/or plasma membrane GLUT4 protein concentration. Sprague Dawley rats were perfused with a perfusate containing no insulin (BAS), a submaximal insulin concnetraiton (sINS), an amino acid mixture with no insulin (AA) or an amino acid mixture with a submaximal insulin concentration (AA-sINS). Following the 25 min perfusion period rats were sacrificed and the gastrocnemius excised. Rates of skeletal muscle glucose uptake were significantly increased in both insulin-stimulated treatments, compared to the non-insulin-stimulated treatment groups. Glucose uptake was greater in the AA-sINS treatment group compared to sINS. However, under non-insulin-stimulated conditions, the addition of amino acids to the perfusate had no impact on glucose uptake compared to BAS. The fact that the amino acid mixture alone did not increase glucose uptake is in contrast to *in vitro* studies demonstrating that amino acids enhance glucose uptake in the absence of insulin (1, 5, 6). These differences are likely due to differences in methodology and the muscles used to compare rates of glucose uptake.

Because the activation of PI 3-kinase is an important signal in insulin-stimulated GLUT4 translocation, both the insulin receptor-1 (IRS-1) associated and total PI 3-kinase activity were determined. Previous *in vitro* studies suggest that PI 3-kinase is required for amino acid-induced glucose uptake (1, 6) but it was not clear from these studies whether the IRS-1 associated PI 3-kinase or another class of PI 3-kinase was involved in this process. Insulin significantly increased both IRS-1 associated and total PI 3-kinase activity compared to when no insulin was in the perfusate. But there was no difference in

IRS-1 associated PI 3-kinase activity or total PI 3-kinase activity when the amino acid mixture was added to the perfusate in the presence or absence of insulin. This finding suggests that PI 3-kinase may play only a permissive role in amino acid-stimulated glucose uptake and that amino acids enhance glucose uptake via a mechanism independent of PI 3-kinase.

AS160, a distal signal in the insulin signaling cascade, prevents GLUT4 translocation. However, once phosphorylated, its inhibitory effect on GLUT4 translocation is removed and GLUT4 is able to move from an intracellular storage pool to the plasma membrane. In Study 2, insulin-stimulation resulted in a significant increase in AS160 phosphorylation compared to the non-insulin-stimulated condition. Furthermore, the addition of the amino acid mixture to the perfusate had a significantly greater effect on AS160 phosphorylation compared to the absence of amino acids under insulin-stimulated conditions. This same phenomenon was not observed when amino acids were added to the perfusate in the absence of insulin. These findings suggest that amino acids act either directly or indirectly to increase glucose uptake by increasing AS160 phosphorylation and possibly GLUT4 translocation to the plasma membrane.

Glucose uptake across the muscle is directly related to the number of GLUT4 transporters at the plasma membrane (3). Because GLUT4 likely had returned to the cytosol by the time muscle was excised from the rats post supplementation, the impact of the amino acid mixture on GLUT4 translocation could not be determined in Study 1. However, theses studies found that intracellular signaling was improved by the amino acid mixture, providing evidence that GLUT4 translocation may have been improved as well. In Study 2, insulin-stimulation resulted in a significant elevation in plasma membrane GLUT4 protein concentration compared to the non-insulin-stimulated treatment groups. There was no difference in plasma membrane GLUT4 in the AA group

compared to BAS but the concentration of GLUT4 at the plasma membrane was significantly greater for AA-sINS compared to the sINS treatment group.

In conclusion, Study 2 found that the amino acid mixture increased glucose uptake when it was perfused with insulin. But in the absence of insulin, the amino acid mixture had no additional impact on glucose uptake. The amino acid-stimulated glucose uptake was no due to increased IRS-1 associated PI 3-kinase activity. However, the phosphorylation status of AS160 and plasma membrane associated GLUT4 protein concentration mirrored the results of glucose uptake. These findings suggest that the amino acid mixture increases AS160 phosphorylation and enhances GLUT4 translocation to the plasma membrane and this was likely the mechanism for the increased amino acid-stimulated skeletal muscle glucose uptake.

STUDY 3

The purpose of Study 3 Exp-1 was to determine if the amino acid mixture lowered the blood glucose response to an oral glucose challenge in insulin resistant obese Zucker rats. Briefly, obese Zucker rats were orally gavaged with either carbohydrate (OB-CHO), carbohydrate plus a 5 amino acid mixture (OB-CHO-AA-1), carbohydrate plus a 5 amino acid mixture with increased leucine concentration (OB-CHO-AA-2) or placebo (OB-PLA). Blood was collected throughout the OGTT as described above for Study 1 Exp-1. There was no difference in fasting blood glucose between treatment groups. Once again, there was very little change in blood glucose throughout the OGTT in the PLA group, suggesting that the rats were not under stress during this procedure. There was a similar peak increase in blood glucose 15 min after supplementation among OB-CHO, OB-CHO-AA-1 and OB-CHO-AA-2 groups. However, beyond the 15 min time point blood glucose was significantly lower for both amino acid mixtures compared to OB-CHO.

The trend for lower blood glucose for the amino acid mixtures would remain for the duration of the OGTT. There was also no statistical difference in blood glucose at any time point between OB-CHO-AA-1 and OB-CHO-AA-2, suggesting that there may be a threshold as to the amount of leucine that is optimal for lowering blood glucose. The glucose AUC showed the amino acid mixture treatment groups to be similar. However, the AUC further displayed the dramatic decrease in blood glucose for the amino acid mixture treatment groups compared to OB-CHO. Since the blood glucose response was similar between OB-CHO-AA-1 and OB-CHO-AA-2, the amino acid mixture with the lower leucine concentration (OB-CHO-AA-1) was used as the carbohydrate plus amino acid mixture for the remainder or the Zucker studies.

Because insulin is primarily responsible for controlling blood glucose the plasma insulin levels were assessed to see if this hormone could explain the differences observed in blood glucose during the OGTT. Fasting blood glucose was not different among the four treatment groups. There were no statistical differences in insulin levels between OB-CHO, OB-CHO-AA-1 and OB-CHO-AA-2 at any time point during the OGTT. However, there appeared to be a trend toward higher insulin levels in the amino acid mixture treatment groups so the insulin AUC was calculated. The AUC showed that plasma insulin was greater in both amino acid mixture treatment groups compared to OB-CHO. This finding would suggest that the lower blood glucose in the animals treated with either amino acid mixture could be, at least partially, explained by enhanced insulin secretion. It is noteworthy that the insulin AUC for the obese Zucker rats, but not the HSD rats, was statistically significant between the carbohydrate and amino acid mixtures. This finding is likely a result of the different animal models being tested. Thus, it is feasible that HSD rats, which are not insulin resistant, do not require elevated insulin for further improvements in glucose tolerance. In contrast, for the insulin resistant obese

Zucker rats, it is possible that a greater insulin response to the amino acid mixture is required to improve glucose tolerance.

The purpose of Study 3 Exp-2 was to determine if the lowered improved insulin resistance observed in Study 3 Exp-1 was due to increased skeletal muscle glucose clearance. This study also sought to determine whether differences in blood glucose uptake could be attributed to changes in protein signaling. Because the tail of a Zucker rat is dark colored, making it difficult to visualize a tail vein, a jugular catheter was surgically implanted in order to deliver the radiolabeled glucose analogue for the determination of glucose uptake. This is in contrast to the SD rats in which the tail vein was used for infusions but this change in methodology was warranted because infusing via a tail vein in Zucker rats was difficult and did not yield consistent results in the pilot studies.

On the day of testing, obese Zucker rats were orally gavaged (8 ml/kg body weight) with either carbohydrate (OB-CHO) or carbohydrate plus the 5 amino acid mixture (OB-CHO-AA-1) or placebo (OB-PLA). Lean Zucker rats were orally gavaged (8 ml/kg body weight) with carbohydrate (LN-CHO). Fifteen min after the gavage, a bolus containing a radiolabeled glucose analogue was injected syringe via the jugular vein catheter. The tail was cut bleed and blood collected at 0, 17, 27, 37, 47 and 60 min post supplementation and the rat sacrificed after the 60 min blood draw.

There was no difference in fasting blood glucose levels among the obese treatment groups. However, the fasting blood glucose of the lean rats was significantly lower compared to each obese treatment group. The blood glucose response for OB-PLA was crucial to this study as this measurement indicates that the rats were properly familiarized and have recovered from the surgical procedure. The small increase in blood glucose measured for OB-PLA was similar to the changes observed in Study 1 Exp-1,

suggesting that the rats had recovered from surgery and were not stressed by the OGTT procedure. The blood glucose response for LN-CHO was significantly lower compared to OB-CHO and OB-CHO-AA-1 at all time points post supplementation. Blood glucose was also significantly reduced for OB-CHO-AA-1 compared to OB-CHO at 27, 37, 47 and 60 min. The glucose AUC shows a similar trend. OB-PLA was lower than all other treatment groups, and the glucose AUC was reduced in LN-CHO compared to OB-CHO and OB-CHO-AA-1. In addition, the glucose AUC was significantly lower in OB-CHO-AA-1 compared to OB-CHO. These findings once again strongly suggest that the blood glucose response is attenuated by the amino acid mixture.

The lower blood glucose response for OB-CHO-AA compare to OB-CHO to the glucose challenge did not appear to be due to enhanced insulin secretion. There was no difference in fasting plasma insulin between the obese rat treatments. However, fasting insulin was significantly lower in the lean rats compared to obese rats. The insulin for LN-CHO was lower than all obese treatments at each time point during the OGTT. OB-PLA, which was gavaged with distilled water, had little change in insulin over the 1 h OGTT, and their insulin was significantly lower than OB-CHO and OB-CHO-AA at all time points. There was no difference in plasma insulin detected between OB-CHO and OB-CHO-AA 0, 17, 27, 47 or 60 min. When the insulin AUC was calculated there was no difference between OB-CHO and OB-CHO-AA. The insulin AUC was significantly lower for LN-CHO compared to OB-CHO and OB-CHO-AA, but not different from OB-PLA.

Since skeletal muscle is the primary site for blood glucose clearance during an OGTT, skeletal muscle glucose uptake was determined across red and white fiber types. Rates of glucose uptake were significantly enhanced in OB-CHO-AA-1 compared to OB-CHO in the red gastrocnemius but not the white gastrocnemius mucle. Glucose uptake

was significantly lower in OB-PLA compared to all other treatments across fiber types. Interestingly, the glucose uptake in OB-CHO-AA-1 was similar to that of LN-CHO in each fiber type. The glucose uptake data suggest that the lower blood glucose response and improved glucose tolerance in obese Zucker rats during the OGTT may be due to increased skeletal muscle glucose clearance.

In Study 3 Exp-2 it is unlikely that the increased glucose uptake in obese Zucker rats was due to enhanced insulin secretion. As noted above, glucose uptake into the muscle is a coordinated process involving a chain of intracellular proteins. It is the activation of specific proteins which allow for the translocation of GLUT4 to the plasma membrane for the subsequent removal of glucose from the blood. Therefore, the activity of specific proteins known to be involved in the glucose transport processes was determined. The acute ingestion of either, distilled carbohydrate or the amino acid mixture did not result in changes to the total protein content. Furthermore, there was no difference in phosphorylation status between treatment groups for Akt/PKB, mTOR or GS. This finding differs from what was observed in non-insulin resistant Sprague Dawley rats from Study 1 Exp-2 which showed enhanced activation in red muscle for mTOR and GS. However, in agreement with what was found in Sprague Dawley rats, AS160 phosphorylation was increased in the amino acid treatment group in both red and white muscle. This is a significant finding because it is thought that AS160 regulates GLUT4 translocation. This would then suggest that there was greater GLUT4 translocation to the plasma membrane when the amino acid mixture was consumed, providing a potential mechanism to explain the improved glucose tolerance.

In conclusions, results from Study 3 suggest that the amino acid mixture can acutely lower the insulin resistance in obese Zucker rats and this improvement appears due to enhanced skeletal muscle glucose uptake and increased AS160 phosphorylation.

FUTURE DIRECTIONS

This series of studies demonstrated the beneficial effects amino acid supplementation has on glucose tolerance in both healthy and insulin resistant rodent skeletal muscle. These studies have also addressed some of the potential mechanisms through which the amino acids lower blood glucose. However, several areas of investigation are still warranted to fully understand the impact amino acids have on glucose tolerance and its potential as a therapeutic in the fight against insulin resistance and type 2 diabetes mellitus.

Although the present series of experiments were able to answer many questions regarding the impact of amino acids on glucose tolerance and insulin resistance, it also serves as the basis for many new questions. The hindlimb perfusion technique could be used to include specific inhibitors, such as Wortmanin, in the perfusate to assess the importance of PI 3-kinase in amino acid stimulated glucose uptake. Including maximally stimulated concentration of insulin and/or amino acids could be used to determine whether the effects of amino acids and insulin were additive and if the amino acids are working via an alternative signaling pathway. Study 3 perfused a 2 mM concentration of amino acids, thus, it is not clear if a lesser dose would achieve the same effect or if the impact of amino acids would be greater with an increased concentration.

The combined effects of amino acids and muscle contraction are not well understood. It is well established that the stimulatory affects of muscle contraction on glucose uptake are insulin independent, and are mediated by both calcium and AMP-activated protein kinase (AMPK) dependent mechanisms. It does not appear that amino acids work through either calcium or AMPK. Thus, it would be interesting to investigate whether the amino acids and contraction signals interact or are independent of each other.

Expanding investigations using the amino acid mixture to include humans would broaden our understanding of supplementation and ultimately determine the therapeutic potential of amino acids. The potential for the amino acid mixture to serve as a non-pharmacological therapeutic to improve insulin resistance is also not currently known. The present investigation demonstrated that the amino acid mixture can lower insulin resistance on an acute basis. However, whether the same acute benefits can be duplicated with chronic administration is unclear. If the chronic administration of the amino acid mixture did work, then it would be important to determine if the acute and chronic mechanisms were the same or different. The acute administration increased the phosphorylation of key signaling proteins and increased GLUT4 translocation to the plasma membrane. A chronic study could be designed to answer questions regarding the impact of amino acid supplementation on body weight, long-term glycemic control, total protein concentration and muscle glycogen levels.

The effects of the amino acid mixture on exercise performance and recovery is open to future investigations. The components of the amino acid mixture were chosen based on their reported effects on different aspects of carbohydrate metabolism. Theoretically, this amino acid mixture would be an excellent ergogenic aid and recovery supplement. Thus, future studies could investigate questions related to time to fatigue, muscle damage, glycogen synthesis, protein synthesis and immune function. Because its been reported that single amino acids cause gastrointestinal distress in some individuals, it would also be of interest to determine how tolerable an amino acid supplement regime would be. Finally, it is likely that studies would need to address whether the concentrations of the amino acids in the current supplement are adequate, and if their concentrations would need to be adjusted to meet one's dietary, exercise and recovery goals.

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Appendix A: Hindlimb Perfusion

Krebs-Henseleit Buffer (KHB) for BSA Dialysis (15 L)

Reagent	Formula Weight	Concentration(mM)	Amount
NaCl	58.44	116	101.53
KCl	74.56	4.6	5.14
$CaCl_2$-$2H_2O$	147	2.5	5.51
KH_2PO_4	136.1	1.16	2.37
$MgSO_4$-$7H_2O$	246.48	1.16	4.29

Reagents were added in the order listed to 12.5 L ddH_2O stirring on a magnetic plate and brought to volume with ddH_2O.

KHB Stock I (1 L)

Reagent	Formula Weight	Concentration(mM)	Amount (g)
NaCl	58.44	1160	67.79
KCl	74.56	46	3.43
KH_2PO_4	136.1	11.6	1.58
$NaHCO_3$	84.01	253	21.25

Reagents were added in the order listed to 800 ml ddH_2O stirring on a magnetic plate and brought to volume with ddH_2O.

KHB Stock II (1 L)

Reagent	Formula Weight	Concentration(mM)	Amount (g)
$CaCl_2H_2O$	147	25	3.68
$MgSO4{\cdot}H_2O$	246.48	11.6	2.86

Reagents were added in the order listed to 800 ml ddH_2O stirring on a magnetic plate and brought to volume with ddH_2O.

Amino Acid Mixture (50 ml)

Amino Acid	Formula Weight	Concentration(mM)	Amount (g)
Cysteine	121	0.87	5.28
Methionine	149	0.45	3.36
Valine	117	1.14	6.68
Isoleucine	131	144.24	944.8
Leucine	131	1.02	6.68

Add amino acids in the order listed to 45 ml KHB stirring on a magnetic plate and bring to volume with KHB.

Solution Preparation Proceduce

1. Prepare KHB for dissolving BSA (1 L)
    a. 100 ml KHB Stock I
    b. Gas with O2/CO2 (95%/5%) for 45 min
    c. 100 ml Stock II KHB
    d. Bring to volume with ddH2O.
2. Prepare 6% BSA
    a. 90 g BSA
    b. 390 ml gassed KHB
    c. Stir until dissolved
3. Fill (~ 1/3 full) pre-wet dialysis tubing with BSA and clamp both ends of the tubing to prevent leaking.
4. Place dialysis tube into tank with 15 L of KHB for dialysis and stir for 48 h.
5. The day before the perfusion prepare the amino acid mixture.
6. After dialyzing BSA, and on the day of the perfusion, check the BSA concentration using a refractometer.

7. Dilute the BSA to 6% with gassed KHB.
8. Filter 2x through fibroglass filter paper using a vacuum pump.
9. Prepare the washout in 6% BSA (125 ml/rat).

	Treatment Group			
	AA-sINS	**sINS**	**AA**	**BAS**
2 mM Sodium-pyruvate	X	X	X	X
500 μU/ml Insulin	X	X		
2 mM Amino acid mixture	X		X	

10. Prepare the perfusate in 6% BSA (150 ml/rat).

	Treatment Group			
	AA-sINS	**sINS**	**AA**	**BAS**
2 mM Sodium-pyruvate	X	X	X	X
500 μU/ml Insulin	X	X		
2 mM Amino acid mixture	X		X	
6 mM Glucose	X	X	X	X
2 mM Mannitol	X	X	X	X
0.2 μCi/ml ^{3}H-2DG	X	X	X	X
0.15 μCi/ml ^{14}C-Sucrose	X	X	X	X

Calculation for washout volume

# of rats = 1

Flow rate = 6 ml/min

Time = 10 min

Dead space of tubing = 35 ml

6 ml/min x 10 min = 60 ml + 35 ml = 95 ml

Prepare 125 ml washout per rat.

Calculation for Perfusate Volume

# of rats = 1

Flow rate = 4 ml/min

Time = 25 min

Dead space of tubing = 35 ml

4 ml/min x 25 min = 100 ml + 35 ml = 135 ml

Prepare 150 ml perfusate per rat.

Appendix B: Glucose Uptake

Glucose Uptake Procedure

1. Place 80-100 mg of muscle sample in a glass test tube containing 1 ml 1 N KOH.
2. Incubate sample for 15 min at 65° C, vortex, then incubate for an additional 5 min at 65° C until the muscle is dissolved.
3. Add 1 ml of 1 N HCl to each test tube sample and vortex to neutralize the digested sample.
4. For muscle samples, aliquot 300 µl of neutralized muscle to a scintillation vial containing 6 ml of Bio-Safe II counting cocktail.
5. To determine the specific activity of the blood aliquot 200 µl of PCA extract to a glass test tube containing 1 ml of 1 N KOH and 1 ml of 1 N HCl and vortex. To determine the specific activity of the perfusate aliquot 200 µl of perfusate to a glass test tube containing 1 ml of 1 N KOH and 1 ml of 1 N HCl and vortex.
6. Aliquot 300 µl of neutralized blood or perfusate to a scintillation vial containing 6 ml of Bio-Safe II counting cocktail.
7. Put all samples in a scintillation counting rack and place rack in a liquid scintillation counter preset for simultaneous counting of ^{3}H and ^{14}C DPM.

Appendix C: Muscle Homogenization

Muscle Homogenization Buffer (100 ml, pH 7.4)

Reagent	Formula Weigh	Concentration(mM)	Amount (g)
HEPES	238.3	20	0.477
EGTA	380.4	2	0.076
NaF	41.9	50	0.209
KCl	74.6	100	0.746
EDTA	292.2	0.2	0.0074
β-Glycerophosphate	216.0	50	1.08
DTT	154.3	1	0.015
PMSF	174.2	0.1	0.00174
Benzamidine	156.6	1	0.0157
Sodium vanadate	181.9	0.5	0.0092

Add reagents in the order listed to 80 ml ddH_2O stirring on a magnetic plate, pH to 7.4 and bring to volume with ddH_2O.

Homogenization Procedure

1. Weight approximately 150 mg of muscle sample.
2. Dilute muscle 1:8 (wt/vol) in homogenization buffer.
3. Homogenize muscle on ice (3 x 10 sec strokes) with a glass tissue grinder set at 3000 rpm.
4. Centrifuge samples at 14,000 x g for 10 min at 4° C.
5. Aliquot the supernatant into microcentrifuge tubes and store at - 80° C.

Appendix D: Lowery Protein Assay

0.1 N NaOH (1000 ml)

Reagent	Amount
NaOH	4 g

Add NaOH 950 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

2% Sodium Carbonate (1000 ml)

Reagent	Amount
Sodium carbonate	20 g

Add sodium carbonate to 950 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

1% Cupric Sulfate (200 ml)

Reagent	Amount
Cupric sulfate	2 g

Add cupric sulfate to 150 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

2% Sodium Potassium Tartrate (200 ml)

Reagent	Amount
Sodium potassium tartrate	4 g

Add sodium potassium tartrate to 150 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

Protein Assay Procedure

1. Thaw BSA (5 mg/ml) on ice.
2. Dilute samples 1:40 by aliquoting 7 µl of sample to a labeled microcentrifuge tube containing 273 µl of ddH_2O and vortex.

3. Prepared standard curve.

Standard	ddH_2O	Standard Protein (ml)	Protein Concentration (mg/ml)
Blank	1.0	0	0
A	1.8	0.2 BSA Stock	0.5
B	0.2	0.8 of A	0.4
C	0.5	0.5 of B	0.2
D	0.5	0.5 of C	0.1
E	0.5	0.5 of D	0.05
F	0.5	0.5 of E	0.25

4. Make Solution A by adding the following:
    a. 96 ml of 2% sodium carbonate
    b. 2 ml of 2% sodium potassium tartrate
    c. 2 ml of 1% cupric sulfate
5. Add 0.1 ml of each standard or sample to the corresponding glass test tube in dublicate.
6. Add 1 ml of Solution A to each sample and standard test tube except to the blank and vortex.
7. Incubate samples and standards for 10 min at room temperature.
8. Dilute the Folin and Ciocalteu's Phenol Reagent 1:2 by adding 1.5 ml of phenol reagent to 3 ml of ddH_2O.

9. After the 10 min incubation period, add 0.1 ml of phenol solution to each sample and standard test tube and vortex.
10. Incubate samples and standards for 30 min at room temperature.
11. Measure the absorbance of each standard and sample at 750 nm using a spectrophotometer.

Calculation

1. Generate a standard curve from the absorbance of the standards. Using the equation from the standard curve calculate the concentration of each sample.
2. Multiply the protein concentration (as determined by the standard curve) by the dilution factor (ie. 40) for the final protein concentration of each sample (μg/μl).

Appendix E: Plasma Membrane Fractionation

Aussie Buffer I (1 L)

Reagent	Formula Weigh	Concentration(mM)	Amount
HEPES	1 M (pH 7.2)	20	20 ml
EGTA	380.4	2	0.76 g
β-Glycerophosphate	216.0	50	10.8 g
DTT	154.3	1	154.25 mg
Sodium vanadate	181.9	1	183.91 mg
Glycerol	100%	10%	10 ml
Benzamidine	156.6	3	469.83 mg
PMSF	174.2	1	0.174 g
Leupeptin	426.6	10 μM	0.0004 g
Pepstati A	50 mM (in DMSO)	5 μM	100 μl

Add reagents in the order listed to 950 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

Aussie Buffer II (1 L)

Add 1% Triton X to Aussie Buffer I

Plasma Membrane Fractionation Procedure

1. Weight approximately 150 mg of muscle sample.
2. Dilute muscle 1:8 (wt/vol) in Aussie Buffer I.
3. Homogenize muscle on ice (3 x 10 sec strokes) with a glass tissue grinder set at 3000 rpm.
4. Balance samples with Aussie Buffer I and centrifuge at 33,200 rpm for 30 min at 4° C.

5. Remove supernatant and store at - 80° C. This is the cytosolic fraction.
6. Resuspend pellet in 4 x the original muscle weight in Aussie Buffer II.
7. Balance samples with Aussie Buffer II and centrifuge at 11,100 rpm for 10 min at 4° C.
8. Remove the supernatant and store at - 80° C. This is the plasma membrane fraction.

Appendix F: 5'-Nucleotidase Activity

5'-Reaction Cocktail (15 ml)

Reagent	Formula Weight	Concentration(mM)	Amount (g)
Glycine	75.07	50	0.0561
$MgCl_2$	95.21	10	0.0141
AMP	347.22	5	0.0261

Add reagents in the order listed to 10 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

Fiske and SubbaRow Cocktail

Reagent	Amount
Fiske and SubbaRow	1.0 g
ddH_2O	6.3 ml

Add the Fiske and SubbaRow to ddH_2O and vortex until dissolved. Keep this solution covered as it is light sensitive.

Stop Solution-8% TCA (25 ml)

Reagent	Amount
TCA	2 g

Add the TCA to 20 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

5'-Nucleotidase Procedure

1. Add 200 µl of 5'-reaction cocktail to labeled microcentrifuge tubes for the samples (not standards).

2. Set a timer at 45 min and add 10 µl of the plasma membrane or cytosolic fraction to each tube at 30 sec intervals. Gently vortex and place on heat block set at 37° C.
3. At the end of 45 min add 400 µl of 8% TCA to stop the reaction
4. Centrifuge samples at 2500 rpm for 10 min.
5. To a second set of labeled sample tubes add the following:
    a. 300 µl ddH_2O
    b. 200 µl supernatant
    c. 100 µl acid molybdate
    d. Vortex at low speed
6. To the BLANK add the following
    a. 200 µl 5'-reaction cocktail
    b. 300 µl ddH_2O
    c. 100 µl acid molybdate
    d. Vortex at low speed

7. Prepare the standard curve:

Tube #	**Phosphorus Standard (µl)**	**Acid Molybdate (µl)**	**ddH_2O (µl)**	**Final Concentration (µM)**
1	0	100	500	0
2	25	100	475	27.08
3	50	100	450	54.17
4	75	100	425	81.25
5	100	100	400	108.33
6	125	100	375	135.42

8. To all sample tubes, blank and standards and 25 µl of Fiske and SubbaRow cocktail, gently vortex and let stand at room temperature for 10 min.
9. Measure the absorbance of each sample at 660 nm using a spectrophotometer.

Calculation

1. Generate a standard curve from the absorbance of the standards. Using the equation from the standard curve calculate the concentration of each sample.
2. Divide the concentration of each sample by 45 (time in minutes) to get µM/min.
3. Obtain the protein concentration (µg/µl) from the Lowery Protein assay and multiple by 10 to get the total protein concentration in the 10 µl sample.
4. Divide this value by 1000 to get mg of protein.
5. Divide the µM/min value by mg of protein to get µM/min/mg.

Appendix G: Western Blot

30% Acrylamide and 1% Bisacrylamide Mixture (200 ml)

Reagent	Amount
Acrylamide	58 g
Bisacrylamide	2 g

Add reagents in the order listed to 150 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O. Filter solution through Whatman #1 filter paper and store in a dark bottle as this solution is light sensitive. Store at 4° C.

1.5 M Tris, pH 8.8 (500 ml)

Reagent	Amount
Trisbase	90.82 g

Add Trisbase to 400 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O. Adjust pH to 8.8 with concentrated HCl. Store at 4° C.

1.0 M Tris, pH 6.8 (500 ml)

Reagent	Amount
Trisbase	60.57 g

Add Trisbase to 400 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O. Adjust pH to 6.8 with concentrated HCl. Store at 4° C.

10% SDS (100 ml)

Reagent	Amount
SDS	10 g

Add SDS to 80 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O. Filter solution through Whatman #1 filter paper.

20% SDS (100 ml)

Reagent	Amount
SDS	20 g

Add SDS to 80 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O. Filter solution through Whatman #1 filter paper.

10% APS (1 ml)

Reagent	Amount
APS	100 mg

Add APS to 1 ml ddH_2O and vortex until dissolve. Make fresh daily.

0.25% Bromophenol Blue (5 ml)

Reagent	Amount
Bromophenol blue	0.0125 g

Add Bromophenol blue to 5 ml ddH_2O stirring on a magnetic plate.

2x Sample Buffer (50 ml)

Reagent	Amount
1.0 M Tris, pH 6.8	5 ml
Glycerol	10 ml
20% SDS	5 ml
β-Mercaptoethanol	2.25 ml
0.25% Bromophenol blue	1.6 ml

Add reagents in the order listed to 40 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

10x Running Buffer (1 L)

Reagent	Amount
Trisbase	30.28 g
Glycine	144.2 g

Add reagents in the order listed to 800 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

10x TBS pH 7.5 (1 L)

Reagent	Amount
Trisbase	60.05 g
NaCl	87.6 g

Add reagents to 800 ml ddH2O stirring on a magnetic plate and bring to volume with ddH_2O. Adjust pH to 7.8 with concentrated HCl.

Anode I (1 L)

Reagent	Formula Weight	Concentration	Amount
Trisbase	121.1	300 mM	36.33 g
SDS	288.4	0.05%	0.5 g
Methanol	32.0	10%	100 ml
β-Mercaptoethanol	78.1	10 mM	0.78 ml

Add reagents in the order listed to 800 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

Anode II (1 L)

Reagent	Formula Weight	Concentration	Amount
Trisbase	121.1	25 mM	3.025 g
SDS	288.4	0.05%	0.5 g
Methanol	32.0	10%	100 ml
β-Mercaptoethanol	78.1	10 mM	0.78 ml

Add reagents in the order listed to 800 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

Cathode (1 L)

Reagent	Formula Weight	Concentration	Amount
Trisbase	121.1	25 mM	3.025 g
α-amino-hexanoic acid	131.2	40 mM	5.25 g
SDS		0.05%	0.5 g
Methanol	32.0	10%	100 ml
β-Mercaptoethanol	78.1	10 mM	0.78 ml

Add reagents in the order listed to 800 ml ddH_2O stirring on a magnetic plate and bring to volume with ddH_2O.

Western Blot Procedure

1. Assemble the gel apparatus in casing stands according to the manufacturer's instructions.
2. Preparing resolving gel solution (10% resolving gel for 2 gels):
    a. 4 ml ddH_2O
    b. 3.3 ml acrylanide mix

c. 2.5 ml 1.5 M Tris, pH 8.8

d. 0.1 ml 20% SDS

e. 0.1 ml 10% APS

f. 0.004 ml TEMED

g. Plunge solution with a glass pipette to mix

3. Fill the caster ~ 3/4 full then overlay with 200 µl butanol. Allow 1 h for gel to polymerize.
4. After gel polymerization, pour off butanol from the resolving gel and rinse between casting plates with ddH_2O. Dry between casting plates with KimWipes.
5. Prepare stacking gel solution (for 2 gels):
    a. 3.4 ml ddH_2O
    b. 0.83 ml acrylamide mix
    c. 0.63 ml 1.0 M Tris, pH 6.8
    d. 0.05 ml 20% SDS
    e. 0.05 ml APS
    f. 0.005 ml TEMED
    g. Plunge solution with a glass pipette to mix
6. Fill the caster with stacking gel solution then gently put combs into place. Allow 45 min for stacking gel to polymerize.
7. During the stacking gel polymerization, prepare samples.
    a. Thaw samples on ice
    b. Dilute samples 1:2 with 2x sample buffer in labeled microcentrifuge tubes.
    c. Vortex then place in boiling water (~ 100° C) for 5 min
    d. Centrifuge at 14,000 x g for ~ 5 sec
8. Prepare 1x running buffer

a. 895 ml ddH_2O
b. 100 ml 10x running buffer
c. 5 ml 20% SDS

9. After stacking gel has polymerized, carefully remove combs and assemble gel apparatus and electrophoresis chamber according to the manufacturer's instructions. Fill inner chamber with 1x running buffer and outer chamber ~ 1/3 full with 1x running buffer.
10. Load the appropriate amount of sample, standard and molecular weight marker to each gel lane.
11. Electrophoresis at 200 V for 1 h.
12. After the electorphoresis, prepare for the semi-dry transfer.
13. Carefully separate casing plates, cut off stacking gel and place resolving gel in Anode I for at least 10 min.
14. Pre-wet PVDF membranes in methanol for 30 sec then place in Anode I for at least 10 min.
15. Pre-wet filter paper in Anode II and Cathode for at least 10 min.
16. Transfer the proteins from the resolving gel to the membrane according to the manufacturer's instructions.
17. Transfer the proteins at 25 V for 25 min.
18. During the transfer, prepare TTBS solution
    a. 900 ml ddH_2O
    b. 100 ml 10x TBS
    c. 600 µl Tween 20
19. During the transfer prepare 2% non-fat dry milk (NFDM). Prepare 50 ml per membrane. (ie 100 ml for 2 membranes)

a. 7 g NFDM
b. 100 ml TTBS

20. After the transfer is complete, place each PVDF membrane a container with 7% NFDM and block for 1 h with gently agitation.
21. After blocking the membrane, wash 2x, 5 min in 25 ml TTBS
22. Incubate membranes with the appropriate primary antibody overnight at 4° C with gently agitation.
23. After the overnight incubation with the primary antibody, wash membranes 2x, 5 min in 25 ml TTBS.
24. Incubate membranes with the species specific secondary antibody for 1 h at room temperature.
25. After the incubation with the secondary antibody, wash membranes 2x, 5 min in 25 ml TTBS.
26. Visualize protein bands using an ECL detection kit and Bio-Rad ChemiDoc detection system according to the manufacturer's instructions.
27. Quantify the density of bands using Quantity One Analysis software.

Appendix H: Raw Data for Study 1

Study 1: Experiment 1

Blood Glucose (mmol/L)

		Time					
Rat #	**Treatment**	**0**	**15**	**30**	**60**	**120**	**AUC**
12	CHO	4.8	10.7	9.4	7.0	5.7	319.6
15	CHO	5.4	9.6	9.0	8.6	7.4	347.6
21	CHO	4.8	8.7	8.5	6.5	4.8	218.6
23	CHO	5.5	9.1	7.9	7.3	5.8	196.9
27	CHO	4.9	8.2	7.3	6.6	5.3	193.7
29	CHO	5.3	9.0	8.6	6.9	5.8	215.3
3	CHO-AA-1	4.6	8.6	7.3	5.9	5.4	200.9
13	CHO-AA-1	5.7	6.6	6.0	6.0	5.4	25.2
14	CHO-AA-1	5.1	7.9	6.8	6.3	6.0	158.0
19	CHO-AA-1	4.4	8.9	7.9	5.9	4.3	208.3
26	CHO-AA-1	5.1	7.8	7.4	6.5	5.1	157.0
31	CHO-AA-1	4.9	9.8	7.0	5.9	4.6	159.5
5	CHO-AA-2	4.8	8.4	7.1	5.6	5.2	155.0
16	CHO-AA-2	5.7	8.0	6.7	6.5	5.8	201.9
17	CHO-AA-2	3.8	7.4	7.3	5.9	4.2	239.6
24	CHO-AA-2	4.9	8.2	6.1	6.0	5.7	151.0
25	CHO-AA-2	5.2	7.9	6.6	6.4	5.4	134.3
32	CHO-AA-2	5.2	7.6	6.6	5.6	4.5	64.5

10	PLA	4.5	5.8	5.5	5.1	4.2	57.1
18	PLA	4.5	5.2	5.2	4.7	4.4	30.6
20	PLA	5.6	5.9	5.8	5.8	5.1	-33.2
22	PLA	5.3	5.7	5.7	5.4	4.6	-0.8
28	PLA	5.1	6.2	6.2	5.8	4.2	43.4
30	PLA	5.2	5.9	6.0	5.2	4.2	-4.2

Study 1: Experiment 1

Plasma Insulin (pmol/L)

		Time					
Rat #	**Treatment**	**0**	**15**	**30**	**60**	**120**	**AUC**
12	CHO	86.8	399.0	272.3	90.7	56.4	8092.5
15	CHO	60.6	260.1	137.6	70.4	39.9	4492.5
21	CHO	46.2	408.1	201.3	59.2	52.9	9699.4
23	CHO	51.8	415.5	131.6	49.7	48.3	7026.8
27	CHO	47.6	274.1	156.8	76.7	48.0	7123.5
29	CHO	89.3	322.4	200.9	79.1	54.6	4542.4
3	CHO-AA-1	61.6	380.1	253.1	74.6	61.6	9619.9
13	CHO-AA-1	72.1	203.7	83.3	67.2	29.8	747.0
14	CHO-AA-1	79.8	357.4	128.5	49.7	53.6	3091.9
19	CHO-AA-1	35.0	922.6	222.6	60.2	55.0	19267.5
26	CHO-AA-1	83.7	322.4	215.6	94.2	67.6	6496.9
31	CHO-AA-1	35.0	286.0	167.7	50.4	43.1	7683.4

5	CHO-AA-2	99.1	849.8	421.8	95.6	58.1	17142.0
16	CHO-AA-2	45.5	179.2	80.2	40.6	37.5	2263.1
17	CHO-AA-2	35.7	262.9	159.6	78.1	60.6	8810.3
24	CHO-AA-2	47.3	428.1	138.3	56.7	56.0	8477.3
25	CHO-AA-2	74.9	552.7	311.9	109.6	70.7	13918.9
32	CHO-AA-2	22.1	472.5	211.8	58.1	26.6	12789.8
10	PLA	76.3	121.1	98.0	88.6	74.9	1705.5
18	PLA	64.8	48.0	56.7	49.4	43.1	-1807.1
20	PLA	48.0	48.3	36.4	21.7	16.5	-2386.9
22	PLA	62.3	53.9	58.8	36.8	15.4	-2725.5
28	PLA	93.8	148.8	118.3	99.8	58.8	569.3
30	PLA	32.2	21.7	26.3	14.0	14.0	-1632.4

Study 1: Experiment 2

Blood Glucose (mmol/L)

		Time						
Rat #	**Treatment**	**0**	**18**	**25**	**35**	**45**	**60**	**AUC**
4	CHO	4.6	8.7	10.6	12.7	14.1	13.5	442.3
7	CHO	4.7	10.6	11.9	11.2	11.1	10.8	394.9
8	CHO	4.5	10.3	13.1	12.3	11.9	11.2	454.2
11	CHO	4.1	10.3	11.0	10.3	9.7	9.3	368.7
12	CHO	5.2	9.5	11.2	10.7	12.7	13.9	378.8
15	CHO	4.1	11.9	16.6	18.5	19.6	21.1	747.2

5	CHO-AA-1	4.5	10.1	11.3	11.8	11.5	10.0	400.0
6	CHO-AA-1	5.3	10.0	8.5	7.9	7.9	7.7	180.5
9	CHO-AA-1	4.7	8.7	9.5	7.5	7.4	6.8	206.1
10	CHO-AA-1	4.3	8.3	10.7	10.7	10.2	9.2	349.1
13	CHO-AA-1	4.8	10.0	11.0	11.2	11.4	9.8	363.6
14	CHO-AA-1	3.3	6.9	9.8	9.0	8.6	8.2	215.6

Study 1: Experiment 2

Plasma Insulin (pmol/L)

		Time						
Rat #	**Treatment**	**0**	**18**	**25**	**35**	**45**	**60**	**AUC**
4	CHO	59.9	160.3	275.8	337.8	307.3	234.2	8904.1
7	CHO	70.0	328.7	197.1	246.8	260.1	162.4	8270.7
8	CHO	57.4	168.4	179.2	152.6	165.9	103.6	4640.9
11	CHO	56.7	227.5	148.8	132.0	109.9	119.0	4686.6
12	CHO	77.0	99.4	139.0	86.8	180.3	246.4	2740.5
15	CHO	135.5	199.5	289.1	248.2	335.0	272.0	5637.0
5	CHO-AA-1	86.8	302.1	258.0	74.6	90.3	126.0	4759.8
6	CHO-AA-1	202.7	283.2	344.4	395.2	221.2	58.8	3586.5
9	CHO-AA-1	52.2	68.3	49.7	62.7	64.8	85.4	597.8
10	CHO-AA-1	80.9	197.8	381.2	341.3	393.1	268.5	10269.6
13	CHO-AA-1	104.7	273.7	224.0	388.9	202.0	107.5	6900.6
14	CHO-AA-1	83.3	370.7	265.7	238.0	276.2	289.8	9765.4

Study 1: Experiment 2

Skeletal Muscle Glucose Uptake (µmol/g/h)

Rat #	Treatment	RG	WG
4	CHO	3.5	1.0
7	CHO	6.7	1.6
8	CHO	4.4	1.2
11	CHO	5.8	1.1
12	CHO	7.6	1.4
15	CHO	3.7	0.3
5	CHO-AA-1	11.0	2.9
6	CHO-AA-1	9.7	2.2
9	CHO-AA-1	5.2	1.5
10	CHO-AA-1	4.1	1.0
13	CHO-AA-1	12.1	1.2
14	CHO-AA-1	7.9	2.3

Study 1: Experiment 2

Akt/PKB (% of Standard)

		Total Protein		Thr-308 Phosphorylation	
Rat #	Treatment	RG	WG	RG	WG
4	CHO	154.3	93.8	20.5	26.1
7	CHO	129.4	91.7	17.8	27.4

8	CHO	101.7	101.5	30.9	33.6
11	CHO	130.5	104.7	28.8	24.0
12	CHO	138.0	196.4	23.8	76.0
15	CHO	215.2	131.3	42.2	10.6
5	CHO-AA-1	90.8	80.6	20.4	26.9
6	CHO-AA-1	138.3	98.2	16.6	26.2
9	CHO-AA-1	128.3	88.6	32.9	26.3
10	CHO-AA-1	135.4	111.3	40.7	24.6
13	CHO-AA-1	167.3	181.1	26.6	67.3
14	CHO-AA-1	234.3	175.8	59.4	23.8

Study 1: Experiment 2

mTOR (% of Standard)

		Total Protein		**Ser-2448 Phosphorylation**	
Rat #	**Treatment**	**RG**	**WG**	**RG**	**WG**
4	CHO	74.2	65.6	54.5	41.8
7	CHO	82.1	67.8	33.0	44.7
8	CHO	49.5	49.6	30.8	35.5
11	CHO	77.5	41.8	33.8	25.4
12	CHO	72.2	41.1	34.8	28.3
15	CHO	70.8	52.1	37.5	34.4
5	CHO-AA-1	43.6	59.7	52.7	43.6
6	CHO-AA-1	65.9	62.7	43.9	52.5

9	CHO-AA-1	65.3	40.7	51.4	30.5
10	CHO-AA-1	55.1	31.3	55.5	28.5
13	CHO-AA-1	84.6	37.7	52.6	28.8
14	CHO-AA-1	78.3	48.6	65.3	36.6

Study 1: Experiment 2

GS (% of Standard)

		Total Protein		**Ser-641 Phosphorylation**	
Rat #	**Treatment**	**RG**	**WG**	**RG**	**WG**
4	CHO	100.8	90.4	100.6	81.2
7	CHO	88.5	88.3	79.5	66.3
8	CHO	105.5	77.7	58.3	76.4
11	CHO	118.5	100.4	86.3	46.5
12	CHO	116.7	72.3	69.0	65.4
15	CHO	30.1	107.1	80.2	70.1
5	CHO-AA-1	85.7	113.6	56.0	55.3
6	CHO-AA-1	81.5	110.4	51.1	75.2
9	CHO-AA-1	94.3	84.6	49.9	65.2
10	CHO-AA-1	87.0	76.2	40.4	58.1
13	CHO-AA-1	98.2	75.5	72.0	53.2
14	CHO-AA-1	88.5	98.4	17.3	70.1

Study 1: Experiment 2

AS160 (% of Standard)

		Total Protein		Thr-642 Phosphorylation	
Rat #	**Treatment**	**RG**	**WG**	**RG**	**WG**
4	CHO	104.4	111.9	69.9	57.7
7	CHO	82.7	55.1	52.5	38.3
8	CHO	109.8	87.2	70.2	46.1
11	CHO	76.0	77.0	45.2	36.1
12	CHO	131.3	89.4	48.0	42.9
15	CHO	95.0	81.1	26.8	37.8
5	CHO-AA-1	86.7	60.8	58.9	90.8
6	CHO-AA-1	78.8	68.5	63.1	52.6
9	CHO-AA-1	87.1	77.1	81.0	54.7
10	CHO-AA-1	107.4	83.1	75.4	70.1
13	CHO-AA-1	86.4	63.5	54.5	65.6
14	CHO-AA-1	97.3	72.5	57.2	51.3

Appendix I: Raw Data for Study 2

Study 2

Skeletal Muscle Glucose Uptake (µmol/g/h)

Rat #	Treatment	Gastrocnemius
2	AA-sINS	5.7
3	AA-sINS	6.6
4	AA-sINS	5.5
6	AA-sINS	5.0
7	AA-sINS	5.0
10	AA-sINS	6.7
13	AA-sINS	6.0
18	AA-sINS	5.2
19	AA-sINS	5.2
1	sINS	3.4
1A	sINS	4.9
11	sINS	5.3
15	sINS	4.5
17	sINS	4.3
30	sINS	4.0
33	sINS	2.8
5	AA	1.97
8	AA	2.3
12	AA	1.16

14	AA	2.27
16	AA	1.83
21	AA	2.66
22	AA	1.6
9	BAS	1.4
17A	BAS	2.6
20	BAS	2.1
20A	BAS	2.2
24	BAS	1.1
32	BAS	1.0

Study 2

IRS-1 Associated PI 3-Kinase Activity (pmol/mg)

Rat #	Treatment	PI 3-Kinase
2	AA-sINS	2.16
3	AA-sINS	2.17
4	AA-sINS	2.57
6	AA-sINS	2.03
7	AA-sINS	2.47
10	AA-sINS	1.93
13	AA-sINS	2.06
18	AA-sINS	2.30
19	AA-sINS	2.05

1	sINS	2.01
1A	sINS	2.36
11	sINS	2.19
15	sINS	2.06
17	sINS	2.05
30	sINS	2.22
33	sINS	1.85
5	AA	0.58
8	AA	0.48
12	AA	0.55
14	AA	0.74
16	AA	0.51
21	AA	0.79
22	AA	0.87
9	BAS	0.75
17A	BAS	0.57
20	BAS	0.67
20A	BAS	0.41
24	BAS	0.79
32	BAS	0.75

Study 2

Total PI 3-Kinase Activity (pmol/mg)

Rat #	Treatment	PI 3-Kinase
2	AA-sINS	2.99
3	AA-sINS	3.07
4	AA-sINS	4.11
6	AA-sINS	2.71
7	AA-sINS	2.51
10	AA-sINS	3.03
13	AA-sINS	2.92
18	AA-sINS	2.36
19	AA-sINS	3.07
1	sINS	2.71
1A	sINS	4.41
11	sINS	2.70
15	sINS	2.73
17	sINS	2.40
30	sINS	2.71
33	sINS	2.76
5	AA	0.96
8	AA	0.79
12	AA	0.97
14	AA	0.88

16	AA	1.04
21	AA	0.99
22	AA	0.78
9	BAS	0.63
17A	BAS	1.24
20	BAS	0.81
20A	BAS	0.87
24	BAS	0.86
32	BAS	0.73

Study 2

AS160 (% of Standard)

Rat #	Treatment	Thr-642 Phosphorylation
2	AA-sINS	105.42
3	AA-sINS	79.92
4	AA-sINS	102.78
6	AA-sINS	113.54
7	AA-sINS	121.31
10	AA-sINS	92.10
13	AA-sINS	96.99
18	AA-sINS	86.52
19	AA-sINS	80.41
1	sINS	84.69

1A	sINS	69.42
11	sINS	97.64
15	sINS	76.98
17	sINS	99.71
30	sINS	54.15
33	sINS	85.10
5	AA	42.25
8	AA	48.67
12	AA	42.07
14	AA	32.61
16	AA	47.25
21	AA	34.68
22	AA	50.97
9	BAS	39.27
17A	BAS	51.37
20	BAS	52.79
20A	BAS	29.59
24	BAS	32.80
32	BAS	33.20

Study 2

Alpha-Tubulin (% of Standard)

Rat #	Treatment	Alpha-Tubulin
2	AA-sINS	118.18
3	AA-sINS	101.50
4	AA-sINS	99.21
6	AA-sINS	89.63
7	AA-sINS	100.42
10	AA-sINS	100.11
13	AA-sINS	115.16
18	AA-sINS	89.42
19	AA-sINS	87.73
1	sINS	129.32
1A	sINS	76.00
11	sINS	104.19
15	sINS	72.80
17	sINS	90.38
30	sINS	113.53
33	sINS	91.46
5	AA	109.30
8	AA	99.84
12	AA	88.73
14	AA	91.77

16	AA	102.88
21	AA	92.92
22	AA	101.84
9	BAS	122.29
17A	BAS	110.49
20	BAS	83.05
20A	BAS	77.62
24	BAS	91.01
32	BAS	91.74

Study 2

5'-Nucleotidase Activity (umol/min/mg)

Rat #	Treatment	Plasma Membrane	Crude Homogenate	Purity Index
2	AA-sINS	28.3	4.4	6.3
3	AA-sINS	20.2	2.4	8.3
4	AA-sINS	16.6	2.5	6.8
6	AA-sINS	22.9	2.6	8.8
7	AA-sINS	28.1	4.5	6.2
10	AA-sINS	21.9	3.6	6.1
13	AA-sINS	18.9	3.0	6.3
18	AA-sINS	28.6	3.5	8.2
19	AA-sINS	30.7	5.1	6.1

1	sINS	19.6	2.5	7.8
1A	sINS	27.0	3.6	7.6
11	sINS	20.1	2.4	8.5
15	sINS	16.9	3.3	5.1
17	sINS	25.6	3.1	8.2
30	sINS	20.9	3.5	5.9
33	sINS	17.8	2.6	6.9
5	AA	15.0	2.8	5.4
8	AA	18.1	2.2	8.3
12	AA	27.4	3.3	8.3
14	AA	26.6	3.7	7.3
16	AA	27.3	3.0	9.3
21	AA	24.1	3.2	7.4
22	AA	29.6	5.4	5.5
9	BAS	20.7	2.5	8.1
17A	BAS	24.3	3.3	7.4
20	BAS	20.3	2.8	7.3
20A	BAS	19.8	3.5	5.7
24	BAS	21.9	3.3	6.7
32	BAS	22.9	3.2	7.2

Study 2

Plasma Membrane GLUT4 (% of Standard)

Rat #	Treatment	PM GLUT4
2	AA-sINS	70.3
3	AA-sINS	105.2
4	AA-sINS	161.8
6	AA-sINS	72.5
7	AA-sINS	162.3
10	AA-sINS	101.9
13	AA-sINS	91.7
18	AA-sINS	66.0
19	AA-sINS	63.8
1	sINS	44.8
1A	sINS	34.9
11	sINS	64.8
15	sINS	69.6
17	sINS	41.8
30	sINS	60.8
33	sINS	42.0
5	AA	12.2
8	AA	12.5
12	AA	12.7
14	AA	15.2

16	AA	16.9
21	AA	13.3
22	AA	8.9
9	BAS	11.7
17A	BAS	9.1
20	BAS	12.2
20A	BAS	12.6
24	BAS	13.8
32	BAS	13.8

Study 2

Plasma Membrane Na^+K^+-ATPase (% of Standard)

Rat #	Treatment	PM Na^+K^+-ATPase
2	AA-sINS	107.66
3	AA-sINS	112.32
4	AA-sINS	101.2
6	AA-sINS	78.71
7	AA-sINS	84.33
10	AA-sINS	111.9
13	AA-sINS	107.54
18	AA-sINS	104.2
19	AA-sINS	87.55
1	sINS	96.57

1A	sINS	117.37
11	sINS	121.02
15	sINS	81.75
17	sINS	97.43
30	sINS	111.96
33	sINS	105.84
5	AA	107.44
8	AA	86.22
12	AA	93.1
14	AA	114.87
16	AA	110.72
21	AA	130.09
22	AA	82.68
9	BAS	112.33
17A	BAS	76.65
20	BAS	81.94
20A	BAS	93.79
24	BAS	109.71
32	BAS	102.19

Appendix J: Raw Data for Study 3

Study 3: Experiment 1

Blood Glucose (mmol/L)

		Time					
Rat #	**Treatment**	**0**	**15**	**30**	**60**	**120**	**AUC**
2	OB-CHO	5.3	15.1	14.0	12.8	7.5	746.7
4	OB-CHO	5.3	15.7	14.1	10.6	7.9	670.4
8	OB-CHO	6.3	20.1	18.4	13.0	8.7	851.1
13	OB-CHO	5.2	14.2	16.0	9.7	6.3	613.5
20	OB-CHO	6.2	14.2	14.2	11.8	7.0	573.5
22	OB-CHO	5.3	13.0	10.5	8.1	6.3	388.8
25	OB-CHO	6.3	12.3	12.3	11.7	8.6	538.6
32	OB-CHO	5.6	14.7	13.8	9.8	7.4	562.6
34	OB-CHO	5.1	12.5	14.7	11.3	6.1	636.8
5	OB-AA-1	5.7	13.3	12.8	8.2	6.9	422.3
10	OB-AA-1	5.3	14.6	12.9	10.0	7.4	583.4
11	OB-AA-1	5.8	16.1	14.0	6.8	6.3	396.5
16	OB-AA-1	6.1	14.1	9.9	8.4	7.7	356.5
18	OB-AA-1	5.2	13.6	10.4	7.9	6.6	405.8
23	OB-AA-1	5.3	12.9	12.1	6.5	6.1	343.8
27	OB-AA-1	6.1	12.3	9.8	6.7	6.2	204.3
31	OB-AA-1	6.3	10.6	9.8	7.3	6.7	198.4
37	OB-AA-1	6.0	16.0	15.6	9.9	6.9	567.7

3	OB-AA-2	5.3	14.8	13.8	11.4	6.9	656.5
6	OB-AA-2	5.8	14.5	16.2	11.1	7.1	641.7
7	OB-AA-2	6.1	15.3	13.1	8.5	7.0	428.2
15	OB-AA-2	5.5	12.8	12.0	6.3	6.5	320.4
19	OB-AA-2	5.9	15.7	13.6	11.5	7.4	617.8
21	OB-AA-2	5.8	13.0	10.4	8.4	7.2	368.6
28	OB-AA-2	4.9	12.4	9.8	4.8	5.1	223.0
30	OB-AA-2	5.4	13.2	12.5	8.7	6.5	459.5
36	OB-AA-2	5.8	12.4	11.1	6.8	5.9	265.0
1	PLA	5.8	10.9	10.1	8.2	8.2	353.4
12	PLA	5.3	8.6	9.8	9.3	8.1	415.3
14	PLA	5.0	5.8	5.9	5.3	5.2	52.7
17	PLA	5.7	7.4	7.3	6.5	6.3	116.4
26	PLA	5.0	5.4	5.6	5.5	5.4	51.7
29	PLA	6.4	6.4	6.4	6.5	6.5	11.2
33	PLA	5.3	7.0	6.4	6.1	6.2	111.9
35	PLA	4.8	5.5	5.6	5.6	5.5	84.0

Study 3: Experiment 1

Plasma Insulin (pmol/L)

		Time					
Rat #	**Treatment**	**0**	**15**	**30**	**60**	**120**	**AUC**
2	OB-CHO	770.0	2738.8	1461.3	1111.3	901.3	64378.1

4	OB-CHO	787.5	2660.0	1111.3	603.8	848.8	28940.6
8	OB-CHO	945.0	4313.8	2030.0	787.5	1219.8	76098.8
13	OB-CHO	1548.8	6982.5	4418.8	1680.0	1277.5	143850.0
20	OB-CHO	805.0	3981.3	2283.8	962.5	721.9	85509.4
22	OB-CHO	761.3	5171.3	2213.8	1071.9	743.8	112284.4
25	OB-CHO	1121.8	3551.6	3276.9	1464.8	1156.8	101423.4
32	OB-CHO	793.6	2790.4	2599.6	848.8	661.5	69103.1
34	OB-CHO	1564.5	5950.0	2091.3	1519.2	1295.0	67510.9
5	OB-AA-1	1291.5	5075.0	3473.8	945.0	1127.0	85325.6
10	OB-AA-1	861.9	2240.0	2056.3	1394.8	990.5	75383.4
11	OB-AA-1	862.8	5066.3	2366.0	765.6	816.4	91113.8
16	OB-AA-1	826.0	6578.3	2586.5	1384.3	1509.4	171517.5
18	OB-AA-1	503.1	7611.6	825.1	1780.6	618.6	174825.0
23	OB-AA-1	710.5	3809.8	2086.0	650.1	728.0	75245.6
27	OB-AA-1	1391.3	7787.5	5582.5	1753.5	1132.3	198778.1
31	OB-AA-1	1015.0	4151.0	2485.0	889.0	1091.1	76728.8
37	OB-AA-1	942.4	9187.5	5188.8	1531.3	908.3	244695.9
3	OB-AA-2	829.5	2309.1	1727.3	850.5	855.8	44126.3
6	OB-AA-2	1417.5	4033.8	4051.3	2353.8	1120.0	131709.4
7	OB-AA-2	1075.4	4375.0	2450.0	787.5	892.5	61982.8
15	OB-AA-2	784.9	5950.0	3570.0	840.0	848.8	144539.1
19	OB-AA-2	761.3	4033.8	1960.0	892.5	778.8	82490.6
21	OB-AA-2	1207.5	6326.3	2301.3	1426.3	997.5	104934.4
28	OB-AA-2	968.6	11987.5	7097.1	1162.9	738.5	305011.9

30	OB-AA-2	904.8	7291.4	3447.5	1334.4	1729.0	197071.9
36	OB-AA-2	843.5	4477.4	3590.1	1020.3	914.4	126387.2
1	PLA	568.8	560.0	778.8	448.9	550.4	-1351.9
12	PLA	420.0	192.5	542.5	315.0	341.3	-7743.8
14	PLA	752.5	988.8	857.5	603.8	757.8	-630.0
17	PLA	630.0	936.3	840.0	656.3	612.5	9975.0
26	PLA	1052.6	1232.9	1229.4	924.0	742.9	-8400.0
29	PLA	1173.0	1185.6	1272.3	1161.1	883.8	-6789.4
33	PLA	1037.8	1341.4	1261.8	1180.4	1092.0	17640.0
35	PLA	1581.1	1409.6	1185.6	1074.5	840.0	-56503.1

Study 3: Experiment 2

Blood Glucose (mmol/L)

		Time						
Rat #	**Treatment**	**0**	**17**	**27**	**37**	**47**	**60**	**AUC**
66	OB-CHO	6.7	12.3	17.9	23.2	23.1	26.6	669.4
70	OB-CHO	4.3	13.5	13.2	12.9	13.9	14.0	471.8
77	OB-CHO	5.2	17.1	16.4	15.3	15.9	15.9	565.8
78	OB-CHO	4.5	18.0	15.6	15.6	14.6	15.4	590.2
81	OB-CHO	5.4	11.9	12.1	12.2	12.5	12.7	352.9
89	OB-CHO	5.1	12.1	10.5	10.8	12.4	12.1	337.0
65	OB-AA-1	5.4	15.6	14.8	13.8	13.9	13.7	469.7
67	OB-AA-1	5.5	11.0	8.6	8.1	7.8	7.8	173.6

71	OB-AA-1	4.9	13.1	10.5	10.3	10.8	11.0	324.6
79	OB-AA-1	6.4	15.4	12.8	11.1	9.8	9.0	290.2
83	OB-AA-1	5.1	10.2	8.9	9.0	8.6	8.5	206.3
84	OB-AA-1	4.8	14.6	11.8	10.7	10.8	10.8	370.6
91	OB-AA-1	6.3	12.2	10.0	10.1	10.3	9.6	221.5
68	OB-PLA	6.9	7.8	7.5	7.2	7.8	7.4	33.6
72	OB-PLA	4.7	5.4	5.6	5.3	5.9	6.0	48.9
73	OB-PLA	5.3	5.8	6.2	6.3	6.5	6.6	46.6
88	OB-PLA	5.1	6.1	5.6	5.8	5.7	5.4	31.5
90	OB-PLA	5.2	7.3	7.2	6.8	6.7	6.6	91.0
74	LN-CHO	4.4	8.2	7.4	7.4	7.8	7.7	169.5
75	LN-CHO	4.4	6.1	8.9	9.0	9.6	9.2	202.6
76	LN-CHO	4.1	10.0	8.6	8.3	9.3	9.4	263.1
85	LN-CHO	4.6	10.4	10.1	10.3	9.9	8.7	276.9
86	LN-CHO	3.8	7.8	6.8	6.5	7.2	7.2	171.5
87	LN-CHO	4.4	9.1	8.1	7.5	6.9	6.0	170.2

Study 3: Experiment 2

Plasma Insulin (pmol/L)

		Time						
Rat#	**Treatment**	**0**	**17**	**27**	**37**	**47**	**60**	**AUC**
66	OB-CHO	2068.5	2224.3	1180.4	1166.4	1296.8	1845.4	-26125.3
70	OB-CHO	706.1	4320.8	650.1	341.3	653.6	1587.3	49712.3

77	OB-CHO	387.6	4202.6	1771.0	762.1	1112.1	1376.4	83839.9
78	OB-CHO	827.8	5259.6	2032.6	952.9	1274.0	1034.3	79604.4
81	OB-CHO	623.9	2044.0	664.1	700.0	857.5	553.9	22567.1
89	OB-CHO	701.8	3698.6	1233.8	1036.0	1100.8	966.0	55426.4
65	OB-AA-1	828.6	2544.5	857.5	1094.6	1317.8	1137.5	33745.7
67	OB-AA-1	410.4	2954.0	1618.8	1488.4	982.6	516.3	64471.8
71	OB-AA-1	846.1	2646.0	859.3	633.5	1190.0	525.0	24170.6
79	OB-AA-1	1728.1	4071.4	987.0	679.0	1813.0	1219.8	11403.0
83	OB-AA-1	644.9	6835.5	2337.1	1509.4	993.1	903.9	114829.3
84	OB-AA-1	945.0	4109.0	1830.5	947.6	1259.1	1636.3	59700.8
91	OB-AA-1	1429.8	2483.3	1117.4	922.3	1043.0	2170.0	6387.5
68	OB-PLA	995.8	1048.3	1280.1	1552.3	962.5	1078.9	9275.4
72	OB-PLA	609.9	1355.4	1015.0	787.5	686.9	1265.3	21037.2
73	OB-PLA	495.3	757.8	661.5	766.5	1125.3	787.5	17063.4
88	OB-PLA	687.8	678.1	352.6	399.9	615.1	663.3	-7354.4
90	OB-PLA	806.8	898.6	595.0	463.8	518.0	1190.0	-5136.7
74	LN-CHO	10.5	209.1	89.3	94.5	100.6	111.1	5999.4
75	LN-CHO	85.8	290.5	207.4	192.5	242.4	250.3	7918.3
76	LN-CHO	44.6	483.0	167.1	199.5	326.4	237.1	13183.2
85	LN-CHO	43.1	459.4	310.3	298.7	224.7	272.0	14426.2
86	LN-CHO	43.1	459.4	204.2	145.4	280.9	183.2	11902.0
87	LN-CHO	48.3	1190.9	938.2	578.0	216.8	268.8	32992.1

Study 3: Experiment 2

Skeletal Muscle Glucose Uptake (umol/100 g/min)

Rat #	Treatment	RG	WG
66	OB-CHO	2.8	0.5
70	OB-CHO	4.3	0.3
77	OB-CHO	4.3	0.4
78	OB-CHO	3.2	0.3
81	OB-CHO	4.2	0.4
89	OB-CHO	4.2	0.5
65	OB-AA-1	3.9	0.4
67	OB-AA-1	6.4	0.3
71	OB-AA-1	5.0	0.4
79	OB-AA-1	5.1	0.7
83	OB-AA-1	5.6	0.3
84	OB-AA-1	5.0	0.2
91	OB-AA-1	5.1	0.4
68	OB-PLA	0.4	0.3
72	OB-PLA	0.3	0.3
73	OB-PLA	0.9	0.2
88	OB-PLA	1.0	0.1
90	OB-PLA	1.0	0.2
74	LN-CHO	5.9	0.3
75	LN-CHO	5.9	0.3

76	LN-CHO	4.0	0.3
85	LN-CHO	5.5	0.8
86	LN-CHO	4.3	0.3
87	LN-CHO	5.6	0.4

Study 3: Experiment 2

Akt/PKB (% of Standard)

		Total Protein		**Thr-308 Phosphorylation**	
Rat #	**Treatment**	**RG**	**WG**	**RG**	**WG**
66	OB-CHO	160.0	130.1	19.9	35.2
70	OB-CHO	149.0	91.3	34.4	39.2
77	OB-CHO	162.1	136.2	18.0	26.5
78	OB-CHO	141.9	97.2	32.7	23.3
81	OB-CHO	161.2	104.0	20.3	68.2
89	OB-CHO	152.7	134.4	16.6	42.5
65	OB-AA-1	178.1	127.1	33.5	36.4
67	OB-AA-1	140.4	76.4	11.2	26.6
71	OB-AA-1	136.2	107.1	30.7	29.3
79	OB-AA-1	156.9	101.7	17.7	50.7
83	OB-AA-1	151.0	130.1	16.6	22.6
84	OB-AA-1	171.6	131.7	24.8	21.3
91	OB-AA-1	155.8	112.8	38.2	50.8
68	OB-PLA	180.2	89.6	24.6	27.7

72	OB-PLA	170.8	111.5	27.4	43.1
73	OB-PLA	168.6	99.1	36.1	52.3
88	OB-PLA	164.0	90.7	23.2	26.2
90	OB-PLA	166.9	145.4	28.4	22.9
74	LN-CHO	165.6	73.6	50.2	21.1
75	LN-CHO	180.2	95.3	26.5	27.9
76	LN-CHO	149.4	119.3	18.0	22.0
85	LN-CHO	146.4	110.4	26.2	52.3
86	LN-CHO	180.0	132.4	20.5	58.3
87	LN-CHO	158.4	115.1	37.2	28.9

Study 3: Experiment 2

mTOR (% of Standard)

		Total Protein		**Ser-2448 Phosphorylation**	
Rat #	**Treatment**	**RG**	**WG**	**RG**	**WG**
66	OB-CHO	94.9	123.3	24.5	16.7
70	OB-CHO	105.0	93.0	42.2	37.8
77	OB-CHO	93.6	80.7	38.4	17.6
78	OB-CHO	81.9	85.1	41.7	33.6
81	OB-CHO	112.6	79.1	48.3	13.0
89	OB-CHO	93.5	114.5	29.8	20.3
65	OB-AA-1	104.1	91.9	39.6	30.7
67	OB-AA-1	108.8	117.3	36.0	25.9

71	OB-AA-1	102.9	118.4	37.0	22.7
79	OB-AA-1	112.6	100.1	44.5	18.4
83	OB-AA-1	93.0	70.9	42.7	38.8
84	OB-AA-1	112.8	105.1	70.7	13.9
91	OB-AA-1	90.2	123.7	37.0	13.5
68	OB-PLA	109.2	101.6	49.2	23.3
72	OB-PLA	90.5	156.7	42.6	21.6
73	OB-PLA	106.4	66.3	37.5	16.0
88	OB-PLA	104.9	96.9	38.9	20.9
90	OB-PLA	82.5	81.0	33.7	31.4
74	LN-CHO	123.7	86.4	44.4	16.8
75	LN-CHO	103.7	112.7	27.2	23.8
76	LN-CHO	43.1	105.2	15.2	23.5
85	LN-CHO	98.3	101.2	41.1	17.3
86	LN-CHO	109.6	103.6	35.6	19.7
87	LN-CHO	115.9	75.1	63.9	51.5

Study 3: Experiment 2

GS (% of Standard)

		Total Protein		**Ser-641 Phosphorylation**	
Rat #	**Treatment**	**RG**	**WG**	**RG**	**WG**
66	OB-CHO	140.5	86.9	200.1	151.2
70	OB-CHO	132.8	68.0	144.5	51.3

77	OB-CHO	115.5	96.0	109.5	61.9
78	OB-CHO	145.0	95.8	170.5	109.2
81	OB-CHO	129.5	73.7	155.6	112.4
89	OB-CHO	115.9	109.3	139.4	145.7
65	OB-AA-1	117.7	58.0	154.0	73.6
67	OB-AA-1	129.2	66.0	178.5	80.2
71	OB-AA-1	123.3	69.0	158.0	111.2
79	OB-AA-1	158.4	59.0	116.7	87.5
83	OB-AA-1	141.9	125.5	177.2	116.2
84	OB-AA-1	129.4	115.1	148.6	110.7
91	OB-AA-1	92.9	56.6	62.0	61.1
68	OB-PLA	159.8	59.4	155.1	87.9
72	OB-PLA	145.4	85.7	102.6	73.7
73	OB-PLA	111.7	107.8	212.8	119.6
88	OB-PLA	116.2	80.1	126.2	132.1
90	OB-PLA	114.8	103.9	53.6	126.3
74	LN-CHO	138.1	88.0	196.0	122.6
75	LN-CHO	111.8	63.1	121.7	83.5
76	LN-CHO	112.6	80.0	130.5	118.9
85	LN-CHO	117.6	79.4	183.6	106.6
86	LN-CHO	129.0	103.3	141.2	121.2
87	LN-CHO	86.0	82.5	126.1	100.6

Study 3: Experiment 2

AS160 (% of Standard)

		Total Protein		Thr-642 Phosphorylation	
Rat #	**Treatment**	**RG**	**WG**	**RG**	**WG**
66	OB-CHO	115.0	76.1	188.5	112.2
70	OB-CHO	105.1	120.4	63.8	114.7
77	OB-CHO	84.2	122.2	107.8	136.6
78	OB-CHO	96.2	52.5	117.1	129.8
81	OB-CHO	121.0	76.6	124.5	112.8
89	OB-CHO	95.3	129.9	133.5	138.7
65	OB-AA-1	93.0	109.9	165.3	141.8
67	OB-AA-1	91.7	92.7	192.6	103.9
71	OB-AA-1	83.2	107.4	135.3	149.9
79	OB-AA-1	108.8	99.5	219.1	117.8
83	OB-AA-1	112.9	82.3	93.3	102.8
84	OB-AA-1	99.9	131.0	245.4	151.9
91	OB-AA-1	99.5	125.1	120.1	152.4
68	OB-PLA	89.0	100.6	87.2	90.6
72	OB-PLA	104.2	83.3	108.0	95.4
73	OB-PLA	109.5	65.5	64.6	78.1
88	OB-PLA	124.1	129.3	59.8	100.4
90	OB-PLA	104.5	97.5	64.7	210.3
74	LN-CHO	115.1	73.3	128.2	136.8

75	LN-CHO	114.5	105.5	132.4	114.3
76	LN-CHO	124.9	116.9	143.9	140.4
85	LN-CHO	105.1	80.1	208.0	108.2
86	LN-CHO	100.9	138.7	276.0	146.8
87	LN-CHO	108.1	126.5	229.0	135.3

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Vita

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